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<b>(54) Title:</b> DNA ENCODING THE T CELL SURFACE PROTEIN T4 AND USE OF FRAGMENTS OF T4 IN THE TREATMENT OF AIDS			

**(57) Abstract**

A single-stranded nucleic acid molecule which encodes an amino acid sequence comprising at least a portion of a T4 glycoprotein. Additionally, amino acid sequences which comprise at least a portion of a T4 glycoprotein and are useful as prophylaxis for treating a subject with acquired immune deficiency syndrome. These amino acid sequences, which are capable of specifically forming a complex with a human immunodeficiency virus envelope glycoprotein and which are soluble in an aqueous solution may be administered to a subject infected with a human immunodeficiency virus so as to block the human immunodeficiency virus from binding to T4<sup>+</sup> cells. Monoclonal antibodies directed to the water-soluble amino acid sequences of the present invention may be used as vaccines for immunizing a subject against acquired immune deficiency syndrome.

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Exhibit 3

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**DNA ENCODING THE T CELL SURFACE PROTEIN T4  
AND USE OF FRAGMENTS OF T4 IN THE TREATMENT OF AIDS**

**BACKGROUND OF THE INVENTION**

Within this application several publications are referenced by Arabic numerals within parentheses. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entirety are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The different functional classes of T lymphocytes recognize antigen on the surface of distinct populations of target cells. Helper T cells interact largely with macrophages and B cells; cytotoxic T cells interact with a broader range of antigen-bearing target cells. These cellular recognition events are likely to be mediated by the specific association of surface molecules on both effector and target cells. The surface of T cells is characterized by a number of polymorphic, as well as nonpolymorphic, proteins which are restricted for the most part to T lymphocytes. Although most of these molecules are common to all T cells, two classes of surface proteins consistently differ on the different functional classes of T cells, and these proteins have been implicated in T cell-target cell interactions.

One class of surface molecules distinguishes the major functional subsets of T lymphocytes: the surface

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glycoproteins T4 and T8. Early in thymic development, the glycoproteins T4 and T8 are coexpressed on the surface of thymocytes (1). In the peripheral immune system, the T4 and T8 molecules are expressed on mutually exclusive subsets of T cells and are only rarely expressed on the same cell (2, 3). The T4 molecule is expressed on T cells that interact with targets bearing class II major histocompatibility complex (MHC) molecules, whereas T8-bearing T cells interact with targets expressing class I MHC proteins (4, 5, 6, 7, 8, 9). The T4 population of T lymphocytes contains helper cells, whereas the T8 population contains the majority of cytotoxic and suppressor cells (6, 10). However, rare T4<sup>+</sup> T cells can function as cytotoxic or suppressor cells (6, 10), suggesting that the expression of T4 or T8 is more stringently associated with MHC class recognition than with effector function. The significance of these molecules in T cell-target cell interactions can be demonstrated by studies with monoclonal antibodies. Antibodies directed against specific epitopes of the T4 molecule (or the murine equivalent L3T4) inhibit antigen-induced T cell proliferation, lymphokine release and helper cell function (7, 8, 11, 12, 13). Similarly, monoclonal antibodies directed against T8 (or the murine equivalent Lyt2) inhibit cytotoxic T cell-mediated killing (14, 15). These observations, along with the fact that T4 and T8 do not reveal significant polymorphism, has led to the hypothesis that T4 and T8 recognize nonpolymorphic regions of class II and class I molecules, respectively.

A second class of proteins thought to differ on different effector T cells are the receptors that recognize antigen in association with polymorphic regions of MHC

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molecules (16, 17, 18). The interactions of helper T lymphocytes are largely restricted to antigen-bearing target cells expressing class II MHC proteins, whereas cytotoxic and suppressor T cells are restricted to targets bearing class I MHC molecules (4, 5, 6, 7, 8, 9). These specific interactions may be mediated by the T cell receptor (or receptors) that recognize antigen in the context of specific MHC molecules (17, 18). Thus, the T lymphocyte may have two independent receptors capable of recognizing both constant and polymorphic determinants of MHC proteins, and these receptors may be responsible for specific targeting of functionally distinct populations of T cells.

The human acquired immune deficiency syndrome (AIDS) is characterized by a depletion of  $T4^+$  lymphocytes. As a consequence, T cell-mediated immunity is impaired in AIDS patients, resulting in the occurrence of severe opportunistic infections and unusual neoplasms. AIDS results from the infection of T lymphocytes with a collection of closely related retroviruses (LAV, HTLV-III, or ARV), now termed human immunodeficiency virus (HIV). The range of infectivity of these agents is restricted to cells expressing the T4 glycoprotein on their surface.

Therefore, the T4 glycoprotein may serve not only as a receptor for molecules on the surface of target cells,

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but also as a receptor for the AIDS virus. Monoclonal antibodies directed against T4 block AIDS virus infection of T4<sup>+</sup> cells in vitro. Furthermore, recent studies have demonstrated that when T4<sup>+</sup> T lymphocytes are exposed to AIDS virus, the 110 kd envelope glycoprotein of the virus is associated with the T4 molecule on the host cell. The lymphotropic character of the virus could therefore be explained by the restricted expression of its receptor, T4, in subpopulations of T lymphocytes.

The depletion of T4<sup>+</sup> T lymphocytes in AIDS results in the impairment of the cellular immune response. In addition, AIDS is frequently accompanied by central nervous system (CNS) dysfunction, most often the consequence of a subacute encephalitis. AIDS virus RNA and DNA has been identified in affected brains, and virus has been isolated from both brain and cerebrospinal fluid from patients with neurological disorders. These observations suggest that the AIDS virus infects brain cells and is directly responsible for the CNS lesions observed in AIDS patients. Thus, the AIDS virus may be neurotropic as well as lymphotropic. It is therefore important to determine whether T4 is also expressed in the CNS or whether additional brain-specific surface molecules may serve as a receptor for the AIDS virus.

The elucidation of the specific interactions of T4 and T8 would be facilitated by the isolation of the T4 and T8 genes, the determination of their structure, and the ability to introduce them into different cellular environments. The isolation and sequence of a cDNA encoding the T8 molecule has recently been reported

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(19, 20, 21). The deduced protein sequence indicates that T8 is a membrane-bound glycoprotein with an N-terminal domain that bears homology to the variable region of immunoglobulin light chains.

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SUMMARY OF THE INVENTION

The present invention provides a single-stranded nucleic acid molecule which encodes an amino acid sequence comprising at least a portion of a T4 glycoprotein. Also provided is an amino acid sequence comprising at least a portion of a T4 glycoprotein. This amino acid sequence may be capable of specifically forming a complex with a human immunodeficiency virus envelope glycoprotein. In addition to its capability to specifically form a complex with a human immunodeficiency virus envelope glycoprotein, the amino acid sequence may be soluble in an aqueous solution.

The soluble amino acid sequence of the present invention may be used as a therapeutic agent, i.e. a prophylaxis, for the treatment of a subject infected with a human immunodeficiency virus. Moreover, a monoclonal antibody directed to the soluble amino acid sequence of the present invention may be useful as a vaccine for immunizing a human subject against a human immunodeficiency virus. Additionally, a monoclonal antibody directed against the soluble amino acid sequence of the present invention may be useful for preparing T4 glycoprotein anti-idiotypic antibodies. These T4 glycoprotein anti-idiotypic antibodies may be useful as a prophylaxis for treating a subject infected with a human immunodeficiency virus.

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BRIEF DESCRIPTION OF THE FIGURESFigure 1. Cytofluorographic Patterns of Indirect Immunofluorescent Staining with OKT<sup>4</sup> and CKT<sup>8</sup>

Cells ( $5 \times 10^5$ ) were incubated with the mouse monoclonal antibodies OKT<sup>4B</sup> or CKT<sup>8</sup>, washed, and then incubated with FITC-conjugated goat anti-mouse immunoglobulin. The cells were analyzed on a FACS Cell Sorter and plotted by a VAX 11/780 computer as cell number vs. log fluorescence. Untransformed NIH 3T3 cells and L cells gave identical cytofluorographic tracings. Pro 2.2 is a leukemic T cell line with phenotype T3<sup>-</sup>; T4<sup>+</sup>; T8<sup>+</sup>; T11<sup>+</sup>. LTD-4 is a T4<sup>+</sup> primary T cell transformant obtained following transfer of total genomic DNA. 3A+ is an NIH 3T3 cell line that was transformed with the T4-pMV6tk/neo retroviral expression construct.

Figure 2. Northern Blot Analysis of RNA Derived from T4<sup>+</sup> and T4<sup>-</sup> L Cells and Human Cells

Three micrograms of poly(A)<sup>+</sup> RNA or 12  $\mu$ g of total RNA (peripheral T cells and thymocytes) were electrophoresed through a 0.8% agarose-formaldehyde gel, blotted onto GeneScreen (New England Nuclear), and probed with a  $^{32}$ P-labeled 0.6 kb T4 cDNA insert. T4<sup>+</sup> cells include LTD-4 (T4<sup>+</sup>, T8<sup>-</sup> L cell transformant), SK-7 T cell hybridoma (T4<sup>+</sup>, T8<sup>-</sup>), CT-CLL leukemia (T4<sup>+</sup>, T8<sup>-</sup>), Pro 2.2 leukemia (T4<sup>+</sup>, T8<sup>-</sup>), T4<sup>+</sup> enriched peripheral T lymphocytes, and human thymocytes. T4<sup>-</sup> cells include untransformed cells, tk7 (T8<sup>+</sup> L cell transformant), HeLa cells, human neuroblastoma cell (IMR), and T8-enriched peripheral T lymphocytes. The human thymocyte lane was exposed four times longer and photographed on high contrast film.

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Figure 3. Restriction Nuclease Maps of pT4B and the T4 Gene, Sequencing Strategy, and Recombinant Vectors

A. Alignment of the Bam HI restriction fragments of pT4B cDNA and the T4 gene. The order of Bam HI fragments in the T4 gene was determined by Southern blot analysis and genomic clone mapping. The alignment of the 5' end of pT4B and the T4 gene is shown by dotted lines, and the shaded region in pT4B corresponds to the coding sequence. The indicated sizes are in kilobases.

B. Sequencing strategy. Arrows indicate length of sequence determined by subcloning fragments into M13 and sequencing by the dideoxy termination procedure (36).

C. Eukaryotic expression vectors. These constructs contain two Moloney murine leukemia virus long terminal repeats (LTRs) whose orientations are indicated by arrows. The pT4B cDNA was subcloned into the Eco RI site of each vector in the orientation indicated. (a) The T4-pVcos7 construct. (b) The T4-pMV6tk/neo construct contains the neomycin phosphotransferase gene fused to the HSV thymidine kinase promoter.

Figure 4. Southern Blot Analysis of DNA from Untransformed and T4+ L Cells and T, B, and Nonlymphoid Human Cells

Ten micrograms of cellular DNAs were digested with Bam HI, electrophoresed through a 0.8% agarose gel, blotted onto GeneScreen, and probed with a nick-translated pT4B cDNA insert. The indicated size markers are in

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5 kilobases. Hybridizing bands of sizes 20 kb, 6.6 kb, 4 kb, 1.8 kb, and 1 kb appear in all human DNAs. DNAs from T4<sup>-</sup>, nonlymphoid origin include untransformed L cells, human fibroblasts (GM), human neuroblastoma cells (NB), and HeLa cells. CB, CP58, and CP94 are 10 DNAs derived from EBV-transformed human B cell lines. LTD-4 is the T4<sup>+</sup> primary L cell transformant. RPMI and HSB2 are T4<sup>-</sup> human T cell leukemic lines; E<sup>+</sup> cells and thymocytes (Thym.) contain T4<sup>+</sup> T cells. CT-CLL, Jurkat (Jurk.), Fao 2.2, CEM, and Molt 4 are T4<sup>+</sup> T cells. gM4 15 is a genomic clone which contains sequences spanning the 3' end of the T4 gene.

15 Figure 5. Immunoprecipitation of the T4 Glycoprotein from NIH 3T3 Cells Transformed with the Retroviral Expression Constructs

20 L-[<sup>35</sup>S]-methionine labeled proteins from two independent NIH 3T3 transformants, peripheral T lymphocytes, and untransformed 3T3 cells were subjected to lentil lectin chromatography to enrich for glycoproteins. 2.5 x 10<sup>6</sup> cpm of each sample was precleared and then 25 immunoprecipitated with CKT\*4 monoclonal antibodies and Protein A-Sepharose. The beads were washed, dissolved in sample buffer, and electrophoresed through a 10% SDS-polyacrylamide gel under reducing (lanes a-d) and nonreducing (lanes e and f) conditions. Lane a, untransformed NIH 3T3 cells. Lane b, T4C2, an NIH 3T3 cell transformed with the T4-pVcos7 construct. Lanes c and e, 3A+, an NIH 3T3 cell transformed with the T4-pMV6tk/neo construct. Lanes d and f, peripheral human T lymphocytes. Relative molecular masses ( $M_r$ ) are given in kilodaltons.

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Figure 6. Nucleotide Sequence of the T4 cDNA and Translated Sequence of the T4 Protein

The nucleotide and predicted amino acid sequences of the cDNA clone pT4B obtained according to the sequencing strategy outlined in Figure 3B. Numbers shown above the amino acid sequence designate amino acid residue positions. The numbers on the right show nucleotide positions. All extracellular cysteines are marked by (•) or (○). The leader sequence (L), variable-like (V), joining-like (J), transmembrane (TM), and cytoplasmic (CYT) regions are indicated by horizontal arrows below the sequence, although the exact boundaries are ambiguous. Two potential N-linked glycosylation sites (Asn-Leu-Thr) are also indicated (CHC).

Figure 7. In Vitro Translation RNA derived from SP6 Transcription

The full length T4 cDNA insert was subcloned into the RNA expression vector pSP65 (Promega Biotec). Linearized plasmid DNA was transcribed with SP6 polymerase (40), and RNA was translated in a wheat germ system (Bethesda Research Laboratories) containing L-[<sup>35</sup>S]-methionine. The *in vitro* translation products were subjected to electrophoresis through a 10% SDS-polyacrylamide gel (lane T4). Bovine pituitary RNA (BP) was used as a control. Relative molecular masses ( $M_r$ ) are given in kilodaltons.

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Figure 8: Schematic diagram of the T4 glycoprotein spanning the cell membrane

T4 consists of four tandem VJ-like domains ( $V_1J_1-V_4J_4$ ), a hydrophobic membrane-spanning segment (shaded area), and a charged cytoplasmic region (CYT). Two potential N-linked glycosylation sites in the extracellular portion are indicated (—). The positions of introns 2-8 in the T4 gene are also marked ( $\Delta$ ).

Figure 9. Alignment of the Variable, Joining, and Transmembrane Regions of T4 with Members of the Immunoglobulin Gene Family

A. Alignment of the variable region amino acid sequence of T4 with a mouse kappa light chain immunoglobulin J606 (66), T8 (20), a human T cell antigen receptor  $\beta$ -chain YT35 (97), and a human  $\gamma$  cell antigen receptor  $\alpha$ -chain HFB-MLT  $\alpha$  (98). The invariant residues in the light chain variable region are included (Inv.) in the alignment. The alignment was performed in order to maximize identities and structural homologies with T4, which appear as boxed residues. The lines below the sequence with letters A, B, C, C', D, E, F, and G indicate the residues which form  $\beta$ -strands (67).  $\beta$ -strand G continues into the J sequence.

B. Alignment of the joining region amino acid sequence of T4 with the consensus J sequences of the T cell antigen receptor  $\gamma$ -chain, immunoglobulin lambda and kappa light chains, and the J sequence of the human T cell receptor  $\alpha$ -chain (99).

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C. Alignment of the transmembrane regions of T4 and an MHC class II chain (100). The putative transmembrane domain (TM) is indicated below the sequence.

5      Figure 10. Restriction nuclease map of the T4 gene in human chromosomal DNA

10     The positions of the 9 exons were determined by genomic clone mapping, Southern blot analysis, and nucleotide sequencing. The leader sequence (L), variable-like (V), joining-like (J), transmembrane (TM), and cytoplasmic (CYT) regions are boxed. The position of the methionine codon surrounded by the initiation consensus sequence is indicated (ATG) at the beginning 15 of the leader exon (L); the termination codon TGA is shown at the end of the second cytoplasmic exon (CYT). The indicated sizes are in kilobases.

20     Figure 11. Recombinant Retroviral Expression Vectors and Construction of Transformed Cells

A. Recombinant retroviral expression vectors. pMV7 contains two directly repeated Moloney murine sarcoma virus long terminal repeats (LTRs) in the orientation indicated by arrows. pMV7 also contains the bacterial neomycin phosphotransferase gene (neo) fused to the HSV thymidine kinase promoter (tk). Full length cDNA inserts encoding T4 (T4B) (7.0) or T8 (T8F1) (20) were subcloned into the Eco RI site in the orientation indicated by arrows, generating T4-pMV7 and T8-pMV7, respectively. The coding sequences are shown as shaded regions. The indicated sizes are in kilobases.

35     B. Retrovirus-Mediated Gene Transfer Strategy.

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Figure 12. The Efficiency of Infection of Naturally-Isolated and Transformed T4<sup>+</sup> Cells

Cells were inoculated with serial 10-fold dilutions of AIDS virus, incubated for 18 hours at 37°C, washed, and plated in microculture. The frequency of infected cultures was determined by an enzyme-linked immunoabsorbent assay (ELISA) 12 days post-infection (46). The results were plotted as % positive cultures vs. log virus dilution. Infectious virus titer (ID-50) is defined as the reciprocal of the dilution at which 50% of the cultures are positive for virus (47). Naturally isolated T4<sup>+</sup> cells include phytohemagglutinin (PHA)-stimulated normal peripheral lymphocytes (●—●) and the T cell line CEM (○—○). T4<sup>+</sup> transfected cell lines include HSB2-T4<sup>+</sup> T cells (▲—▲) and Raji-T4<sup>+</sup> B cells (■—■). The T8<sup>+</sup> transfected cell lines HSB2-T8<sup>+</sup> and Raji-T8<sup>+</sup> (□—□) served as controls in these studies.

Figure 13. Formation of Syncytia in T4<sup>+</sup> HeLa Transformants

A.  $2 \times 10^5$  monolayer HeLa-T4<sup>+</sup> transformants were mixed with  $2 \times 10^4$  AIDS virus-producing H9 cells and incubated at 37°C. Inspection of the cultures after 18 hours revealed that over 90% of nuclei in the monolayer sheet were contained within syncytia.

B. Anti-T4A monoclonal antibody (1:20) was added to the mixed cultures at the time of seeding. Inspection of the cultures after 18 hours revealed a complete absence of cell fusion.

Cultures were photographed at 160 x magnification.

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**Figure 14. Flow Cytometry Analysis of AIDS Virus binding to T4<sup>+</sup> Transformed Cells**

- 5      Column A. Cells ( $5 \times 10^5$ ) were incubated<sup>14</sup> with fluorescein-conjugated anti-T4A (—) or anti-T8 (---) monoclonal antibodies, washed, and analyzed by cytofluorometry.
- 10     Column B. Cells ( $5 \times 10^5$ ) were incubated with buffer (---), or AIDS virus (—), washed, incubated with fluorescein-conjugated anti-AIDS virus antibody, and analyzed by cytofluorometry.
- 15     Column C. Cells ( $5 \times 10^5$ ) were incubated with buffer (---), or with anti-T4A monoclonal antibody followed by AIDS virus (—), or with anti-T8 monoclonal antibody followed by AIDS virus (---). After a wash, fluorescein-conjugated anti-AIDS virus antibody was added and the cells were analyzed by cytofluorometry.

Fluorescence histograms (cell number vs. fluorescence intensity) of each cell line are arranged horizontally.

25     **Figure 15. Northern Blot Analysis of RNA Derived from Human and Mouse Brain, Lymphoid, and Myeloid Cells**

- 30     A. Northern blot analysis of human RNA samples. One microgram of poly(A)<sup>+</sup> RNA from Raji (T4<sup>-</sup> B cell line), U937 (T4<sup>+</sup> monocytic cell line), and Jurkat (T4<sup>+</sup> T cell line), and five micrograms of poly(A)<sup>+</sup> RNA from cerebral cortex, were electrophoresed through a 1% agarose-formaldehyde gel, blotted onto Hybond (Amersham), and probed with a <sup>32</sup>P-labelled T4 cDNA insert, pT4B (70).

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B. Northern blot analysis of mouse RNA samples. Five micrograms of poly(A)<sup>+</sup> RNA from 3T3 cells (fibroblast cell line), forebrain, and hindbrain, and 20 micrograms of total RNA from thymocytes, were electrophoresed through a 1% agarose-formaldehyde gel, transferred onto Hybond, and probed with a <sup>32</sup>P-labelled L3T4 cDNA insert, pL3T4B.

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DETAILED DESCRIPTION OF THE INVENTION

A single-stranded nucleic acid molecule is provided which encodes an amino acid sequence comprising at least a portion of a T4 glycoprotein. In one embodiment of the invention, the nucleic acid molecule encodes an amino acid sequence capable of specifically forming a complex with a human immunodeficiency virus envelope glycoprotein. In another embodiment of the invention, the nucleic acid molecule is at least 90% homologous to a nucleic acid molecule which encodes an amino acid sequence which is at least a portion of a T4 glycoprotein. In still another embodiment of the invention, the nucleic acid molecule encodes an amino acid sequence which, in addition to its capability of specifically forming a complex with a human immunodeficiency virus envelope glycoprotein, is soluble in an aqueous solution. Within this application "aqueous solution" includes, but is not limited to, detergent-free aqueous buffers and body fluids such as blood, plasma and serum. Additionally, "soluble T4" means a fragment of a T4 glycoprotein which is soluble in an aqueous solution. In a further embodiment of the invention, the nucleic acid molecule encodes an amino acid sequence which is at least a portion of a human T4 glycoprotein.

Also provided is a nucleic acid molecule which is complementary to a single-stranded nucleic molecule encoding an amino acid sequence comprising at least a portion of a T4 glycoprotein. This complementary nucleic acid molecule may be labeled with a detectable marker. Such detectable markers are known in the art to which this invention pertains and include detectable enzymes, radiolabeled moieties, fluorescent moieties, and chemiluminescent moieties.

The single-stranded nucleic acid molecule may be a DNA molecule. In one embodiment of the invention, the DNA molecule comprises at least a portion of the genomic DNA molecule represented by the restriction enzyme map shown in Figure 10. In another embodiment of the invention, the single-stranded nucleic acid molecule may be a cDNA molecule which comprises at least a portion of the nucleic acid sequence shown in Figure 6. In a specific embodiment of the invention, the cDNA molecule encodes an amino acid sequence capable of specifically forming a complex with a human immunodeficiency virus envelope glycoprotein and soluble in an aqueous solution. This cDNA molecule comprises at least a portion of the nucleic acid sequence shown in Figure 6.

The present invention further provides an RNA molecule which encodes an amino acid sequence comprising at least portion of a T4 glycoprotein.

A method for detecting a single-stranded nucleic acid molecule encoding an amino acid sequence which is at least a portion of a T4 glycoprotein is provided by the present invention. This method comprises contacting single-stranded nucleic acid molecules with a labeled, single-stranded nucleic acid molecule which is complementary to a single-stranded nucleic acid molecule encoding an amino acid sequence which is at least a portion of a T4 glycoprotein, under conditions permitting hybridization of complementary single-stranded nucleic acid molecules. Hybridized nucleic acid molecules are separated from single-stranded nucleic acid molecules to detect a single-stranded nucleic acid molecule which encodes an amino acid

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sequence which is at least a portion of a T4 glycoprotein. In one embodiment of the invention, the detected single-stranded molecule is a DNA molecule derived from chromosomal DNA. The chromosomal DNA may be derived from lymphoid, myeloid or brain cells. The lymphoid cell may be a T cell or a B cell. Furthermore, the myeloid cell may be a granulocyte site or a macrophage.

The present invention also provides an amino acid sequence which comprises at least a portion of a T4 glycoprotein. In one embodiment of the invention, the amino acid sequence is capable of specifically forming a complex with a human immunodeficiency virus envelope glycoprotein. In another embodiment of the invention, the amino acid sequence is at least 90% homologous to a portion of a T4 glycoprotein and is capable of specifically forming a complex with a human immunodeficiency virus envelope glycoprotein. In yet a further embodiment of the invention, the amino acid sequence which is at least 90% homologous to a portion of a T4 glycoprotein, in addition to its capability of specifically forming a complex with a human immunodeficiency virus envelope glycoprotein, is soluble in an aqueous solution.

Also provided is a peptide which comprises at least one amino acid sequence of the present invention which is a portion of a T4 glycoprotein. A polypeptide which comprises at least two of these peptides is also provided.

In one embodiment of the invention, the amino acid sequence which is capable of specifically forming a complex with a human immunodeficiency virus envelope

glycoprotein and which is soluble in an aqueous solution is useful as a therapeutic agent for the treatment of a subject infected with a human immunodeficiency virus, i.e. as a prophylaxis for AIDS.

5 In a preferred embodiment of the invention, the amino acid sequence comprises the amino acid sequence shown in Figure 6 from at least amino acid -23 to at most amino acid +374. Other preferred embodiments of the invention include amino acid sequences which comprise 10 the amino acid sequence shown in Figure 6 from at least amino acid +287 to at most amino acid +374, from at least amino acid +182 to at most amino acid +286, from at least amino acid +112 to at most amino acid +181, and from at least amino acid +1 to at most amino acid 15 +111.

A pharmaceutical composition useful as a therapeutic agent for the treatment of a subject infected with a human immunodeficiency virus is also provided. This 20 pharmaceutical composition comprises an amino acid sequence of the present invention which is capable of specifically forming a complex with a human immunodeficiency virus envelope glycoprotein and is soluble in an aqueous solution and a pharmaceutically acceptable carrier. Such pharmaceutically acceptable 25 carriers are known in the art to which the present invention pertains and include, but are not limited to, 0.01-0.1M, preferably 0.05 M, phosphate buffer or 0.8% saline.

30 A method for treating a subject infected with a human immunodeficiency virus is also provided. This method comprises administering to the subject an effective amount of a pharmaceutical composition containing a pharmaceutically acceptable carrier and an amino acid 35

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sequence of the present invention, capable of specifically forming a complex with a human immunodeficiency virus envelope glycoprotein and soluble in an aqueous solution, so as to render human immunodeficiency viruses (also referred to herein as AIDS viruses) with which the subject is infected incapable of infecting T4<sup>+</sup> cells.

The present invention also provides a purified polypeptide encoded by a cDNA molecule which comprises at least a portion of the nucleic acid sequence shown in Figure 6.

Further provided is a vector which comprises a cDNA molecule which is at least a portion of the nucleic acid sequence shown in Figure 6. In one embodiment of the invention, the vector comprises a plasmid. In another embodiment of the invention, the vector comprises a virus.

A host vector system for the production of an amino acid sequence which is at least a portion of a T4 glycoprotein is also provided by the present invention. This host vector system comprises a plasmid of the present invention in a suitable host. In one embodiment of the invention, the suitable host is a bacterial cell. In another embodiment of the invention, the bacterial cell is an Escherichia coli cell. In yet another embodiment of the invention, the suitable host a eucaryotic cell. In a further embodiment of the invention, the eucaryotic cell is a mammalian cell. In yet a further embodiment of the invention, the eucaryotic cell is a yeast cell. In still another embodiment of the invention, the suitable host is an insect cell.

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A method for producing a amino acid sequence which is at least a portion of a T4 glycoprotein is further provided. This method comprises growing a host vector system of the present invention under suitable conditions permitting production of at least a portion of a T4 glycoprotein, and recovering the resulting portion of a T4 glycoprotein. The present invention further provides host vector systems and methods for producing an amino acid sequence which is at least a portion of a T4 glycoprotein wherein the vector comprises a cDNA molecule of the present invention and a virus. Suitable hosts include, but are not limited to, bacterial cells, e.g. *Escherichia coli* cells, eucaryotic cells, e.g. mammalian and yeast cells, and insects. An amino acid sequence which is at least a portion of a T4 glycoprotein may be produced by growing a host vector system which comprises a virus and a cDNA molecule of the present invention under suitable conditions permitting production of at least a portion of T4 glycoprotein. The resulting portion of a T4 glycoprotein may be recovered from the host vector system by methods known in the art.

The present invention also provides a substance capable of forming a complex with an amino acid sequence which is capable of specifically forming a complex with a human immunodeficiency virus envelope glycoprotein and is soluble in an aqueous solution. In one embodiment of the invention, the substance is an antibody. In another embodiment of the invention, the antibody is a monoclonal antibody. In yet a further embodiment of the invention, the monoclonal antibody is a human monoclonal antibody.

Also provided is a vaccine useful for immunizing a human subject against a human immunodeficiency virus. This vaccine comprises a monoclonal antibody of the present invention and a pharmaceutically acceptable carrier. By administering to a human subject an effective immunizing amount of a vaccine of the present invention, the production of antibodies capable of neutralizing human immunodeficiency viruses may be invoked, thereby immunizing the subject against a human immunodeficiency virus.

Also provided is a substance capable of specifically forming a complex with a monoclonal antibody of the present invention. In one embodiment of the invention, the substance is capable of additionally forming a specific complex with a human immunodeficiency virus envelope glycoprotein. In a preferred embodiment of the invention, the substance comprises a T4 glycoprotein anti-idiotypic antibody which contains an "internal image" of the T4 binding domain capable of recognizing the receptor binding domain of a human immunodeficiency virus envelope glycoprotein.

A pharmaceutical composition is provided which comprises a T4 glycoprotein anti-idiotypic antibody of the present invention and a pharmaceutically acceptable carrier. Further provided is a method for treating a subject infected with a human immunodeficiency virus by administering to the subject an effective amount of the pharmaceutical composition of the present invention which comprises a T4 glycoprotein anti-idiotypic antibody and a pharmaceutically acceptable carrier so as to render human immunodeficiency viruses with which the subject is infected incapable of infecting  $T4^+$  cells.

5       The various prophylaxis and immunization methods  
AIDS provided by the present invention are based  
the abilities of the novel peptides, antibodies,  
DNA molecules disclosed herein to form complexes w  
or hybridize to, specific molecules and to invoke  
immunological response effective for neutralizing  
AIDS virus. These molecules, methods for t  
preparation, and methods of AIDS treatment will  
better understood by reference to the follo  
experiments and examples which are provided  
purposes of illustration and are not to be construe  
in any way limiting the scope of the present invent  
which is defined by the claims appended hereto.

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Materials and Methods

Cells and Antibodies

5      Peripheral blood leukocytes isolated by Ficoll-Hypaque density gradient centrifugation were fractionated in sheep erythrocyte rosette-positive ( $E^+$ ) cells.  $T4^+$   
10.      $T8^+$  subsets within the  $E^+$  population were isolated by positive selection of  $T8$ -bearing cells with anti-antibody and human erythrocytes conjugated with affinity-purified rabbit anti-mouse IgG (10). Cytofluometric analysis of these subsets demonstrated that  $T4^+$  cells were >95%  $T4^+$  and <2%  $T8^+$ , whereas the  $T8^+$  cells were >95%  $T8^+$  and <2%  $T4^+$ .

15     The Fro 2.2 T cell line ( $T3^-$ ,  $T4^+$ ,  $T8^+$ ,  $T11^+$ ) was derived from an adult patient with undifferentiated acute leukemia. Jurkatt is  $T3^-$ ,  $T4^+$ ,  $T8^+$ ,  $T11^-$ , RPMI 8402  $T3^-$ ,  $T4^-$ ,  $T8^-$ ,  $T11^+$ . CT-CLL is a chronic lymphocytic leukemia which is  $T3^+$ ,  $T4^+$ ,  $T8^-$ , and  $T11^+$  (22).  $T4^+$  cell lines CEM and Molt 4 were obtained from American Type Culture Collection. All leukemic T cell lines were continuously grown in RPMI 1640 medium containing 5% fetal calf serum. Transformed B cell lines CB, CP58 and CP94 were derived as previously described (23).

20     Affinity-purified rabbit anti-mouse IgG was conjugated to human erythrocytes by the chromium chloride method (24).

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Co-transformation of L Cells and NIH 3T3 Cells

Murine L  $tk^{-}aprt^{-}$  cells were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum (Gibco) and 50 micrograms/ml diaminopurine (DAP). L cells were plated out at a density of  $5 \times 10^4$  cells per 10 cm dish, 1 day before transformation. Calcium phosphate precipitates were prepared by the method of Graham and van der Eb (25), as modified by Wigler et al. (26), using 100 ng of pTK and 20 micrograms of high molecular weight T cell or L cell DNA per dish. The L cells were placed under selection in DME with 10% calf serum, 15 micrograms/ml hypoxanthine, 1 microgram/ml aminopterin and 5 micrograms/ml thymidine (HAT medium (27)) on the following day. After 12-14 days of HAT selection,  $tk^{+}$  transformants were screened using the rosetting assay.

Murine NIH 3T3 cells were maintained in DME supplemented with 10% newborn calf serum (Gibco). NIH 3T3 cells were plated out at a density of  $5 \times 10^4$  cells per 10 cm dish, 2 days before transformation. A calcium phosphate precipitate was applied to the cells using 10 micrograms of carrier DNA and either 10 micrograms of T4-pMV6tk/neo or 10 micrograms of T4-pVcos7 and 500 ng of pSV2neo. After 2 days, the cells were placed under selection in DME with 10% calf serum and 500 micrograms/ml G418 (Geneticin®; Gibco). Rosetting assays were performed on surviving colonies one week after growth in selective medium.

Rosetting Assay

After one rinse with phosphate-buffered saline (PBS), the plates were incubated with 2.5 ml of the purified

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monoclonal antibody CKT\*4A (1 mg/ml) diluted at 1/500 in PBS containing 5% fetal calf serum for 45 minutes at room temperature. Free antibody was removed from the plates with three gentle rinses in PBS. Six milliliters of human erythrocytes conjugated with purified rabbit anti-mouse IgG antibody (2% v/v stock suspension, diluted 1/10 in PBS/5% fetal calf serum) were added and the plates were left at room temperature. After 45 minutes, free erythrocytes were gently aspirated and PBS was added prior to inspection for rosette-positive colonies.

#### Cytofluorometric Analysis

15 Adherent cells were removed with 0.005 M EDTA in PBS and washed once with PBS containing 1% bovine serum albumin (BSA) and 0.01% sodium azide (cytowash). Cells ( $5 \times 10^6$ ) in 0.1 ml were added to tubes with appropriate dilutions of CKT\*4, CKT\*8 or control antibodies. The cell-antibody mixture was incubated for 45 minutes at 4°C and then washed twice in cytowash. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG + A + M (Cappel) was added to the cells and incubated for 1 hour at 4°C. The cells were then washed three times in cytowash and resuspended in 0.5 ml of PBS with 0.01% sodium azide. The cells were analyzed on a Becton Dickinson FACS IV Cell Sorter and the data was stored and plotted using a VAX 11/780 computer (Digital Equipment Co.)

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RNA and DNA Isolation

Total RNA was isolated from cells by homogenation in 4 M guanidinium thiocyanate, followed by ultracentrifugation through a 5.7 M CsCl cushion (28). Poly(A)<sup>+</sup> selection was achieved by oligo(dT)-cellulose chromatography (Type 3, Collaborative Research) (29). High molecular weight genomic DNA was prepared as described by Wigler et al. (26).

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cDNA and Genomic Libraries

Double-stranded cDNA was synthesized from poly(A)<sup>+</sup> RNA derived from peripheral human T cells (20). After treatment with EcoRI methylase and T4 DNA polymerase, the double-stranded cDNA was cloned into the EcoRI site of λ gt10 (30) using EcoRI linkers. The Charon 4 human genomic library was generously provided by Dr. Tom Maniatis (Harvard University) (31).

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Synthesis of a Subtracted cDNA Probe

<sup>32</sup>P-labeled cDNA was synthesized from poly(A)<sup>+</sup> RNA derived from the primary transformant, LTD-4, as described by Davis et al. (32). After annealing the cDNA to an excess of untransformed L cell poly(A)<sup>+</sup> RNA (R<sub>O</sub> = 3000), single-stranded sequences, which were enriched for human cDNAs, were isolated by hydroxyapatite chromatography (32). Prior to filter hybridization, the subtracted cDNA probe was concentrated with sec-butanol and desalted on a G-50 Sephadex column equilibrated in TE.

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### Screening of cDNA and Genomic Libraries

The peripheral human T cell library was plated on *E. coli* C600/HFL and the human genomic library was plated on *E. coli* LE392. Screening of duplicate filters was carried out according to the standard procedure (33), with the hybridization performed in 50% formamide and 5x SSC at 42°C. In the screen of the cDNA library, 6 x 10<sup>4</sup> cpm of subtracted probe was applied per 137 mm nitrocellulose filter. Filters from the genomic library were hybridized to a nick-translated (34) cDNA insert. The washes were performed at 68°C, with a final wash in 0.2 x SSC. Autoradiography was performed at -70°C in the presence of intensifying screens for 1-2 days.

### DNA Sequencing

Restriction fragments of pT4B were subcloned into the M13 vectors mpl8 and mpl9 (35). Sequencing reactions were performed using the dideoxy chain termination technique (36). The sequencing strategy is depicted in Figure 3B.

### Southern and Northern Blot Hybridizations

High molecular weight cellular DNAs were digested with 5 units of restriction nuclease per microgram of DNA according to the manufacturer's recommendation (Boehringer Mannheim). Samples (10 micrograms) were subjected to electrophoresis on a 0.8% agarose gel. DNA fragments were transferred to GeneScreen (New England Nuclear; (37)) and hybridized as described by Church and Gilbert (38).

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RNA was run on a 0.8% agarose-formaldehyde gel (39) and transferred to GeneScreen. Northern hybridization was performed according to the procedures supplied by the manufacturer. Both Southern and Northern blots were hybridized to nick-translated probes.

Synthesis and In Vitro Translation of SP6 RNA

10 The kb T4 cDNA was subcloned into the EcoRI site of pSP65 (Promega Biotech) and linearized with HindIII. Transcription of linearized plasmid DNA (1 microgram) with SP6 polymerase in the absence of radiolabeled nucleotides was performed as described (40), except that GpppG and unlabeled CTP were added to the transcription buffer. One-tenth of the reaction mixture was translated in a wheat germ system (Bethesda Research Laboratories) containing L-[<sup>32</sup>S]-methionine (Amersham) and 1 micromolar S-adenosylmethionine. The in vitro translation products were subjected to SDS-polyacrylamide electrophoresis under reducing conditions as described below.

25 Cell Labeling, Lectin Chromatography and Immunoprecipitation

Cells were grown for 12 hours in methionine-free DME medium containing 10% dialyzed calf serum and 1-mCi of L-[<sup>32</sup>S]-methionine (Amersham) as previously described (41). The cells were solubilized in 10 mM Tris (pH 7.4), 150 mM NaCl (TBS) containing 0.5% Nonidet P-40 (Shell) and 0.2 mM phenylmethylsulfonyl fluoride (Sigma). The lysates were centrifuged for 1 hour at 100,000 x g, and the supernatants were subjected to lentil lectin chromatography (Pharmacia) according to the procedures of Hedo et al. (42). Eluates were pre-

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absorbed once with a mixture of control mouse ascites  
and protein A-Sepharose (Pharmacia) for 1 hour at 4°C  
and twice with protein A-Sepharose alone for 1 hour at  
4°C. Cf each supernatant,  $2.5 \times 10^4$  cpm were then  
5 mixed with 10 microliters monoclonal antibody (approximately 1 mg/ml) and protein A-Sepharose and incubated  
on a turntable overnight at 4°C. The beads were then  
washed four times with cold TBS containing 0.5% NP-40  
10 and 0.2% SDS and were resuspended in electrophoresis  
sample buffer.

#### Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed  
15 according to the procedure of Laemmli (43). The immunoprecipitates and in vitro translation products  
were dissolved in sample buffer with or without 2-mercaptoethanol and then were applied to 10% polyacrylamide gels. Autoradiography was performed on  
20 Kodak XAR-5 film in the presence of intensifying  
screens (DuPont Chemical Company).

#### Cotransformation and Rosetting Assay

25 Mouse 3T3 cells (44) were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum (CS) (Gibco). 3T3 cells were plated out at a density of  $5 \times 10^5$  cells per 10 cm dish, 2 days before transformation. Calcium phosphate precipitates  
30 were prepared by the method of Graham and van der Eb (25), as modified by Wigler et al. (27). Precipitates were applied to the cells using 10 micrograms of carrier DNA and either 10 micrograms of T4-pMV7 or 10 micrograms of T8-pMV7. After 2 days, the cells were  
35 placed under selection in DME/10% CS and 500 micrograms/ml G418 (Geneticin®; Gibco).

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5 Rosetting assays to identify T4<sup>+</sup> or T8<sup>+</sup> colonies were performed on surviving colonies 1 week after growth in selective medium. After one rinse with phosphate-buffered saline (PBS), the plates were incubated with 2.5 ml of the purified monoclonal antibody CKT\*4A or CKT\*8 (1mg/ml; Ortho) diluted at 1/500 in PBS containing 5% fetal calf serum (FCS) for 45 minutes at room temperature. Free antibody was removed from the plates with three gentle rinses in PBS. 6 ml of human erythrocytes conjugated with purified rabbit anti-mouse IgG antibody (2% v/v stock suspension, diluted 1/10 in PBS/5% FCS) were added and the plates were left at room temperature. After 45 minutes, free erythrocytes were 10 gently aspirated and PBS was added prior to inspection. 15 T4<sup>+</sup> and T8<sup>+</sup> -2 clones were purified by colony isolation and characterized by flow cytometry and Northern blot analysis.

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#### Recombinant Retrovirus Production and Infection

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T4<sup>+</sup> and T8<sup>+</sup> -2 clones were isolated which produce recombinant retrovirus stocks with titers of 10<sup>5</sup> cfu/ml. Viral stocks were prepared by adding 10 ml of fresh DME/10% CS to a near confluent monolayer of the T4<sup>+</sup> or T8<sup>+</sup> -2 clones. After 24 hours, the medium was removed and filtered through a 0.45 micrometer-filter (Millipore). For infection, 5 x 10<sup>5</sup> cells were incubated with 2 ml of viral supernatant (or a dilution) in the presence of 8 micrograms/ml polybrene (Aldrich). After 3 hours, 8 ml of fresh medium was added. 3 days after infection the cells were reseeded into DME/10% CS containing 500 micrograms/ml G418, grown for 2 weeks, scored for G418<sup>r</sup> colonies, and screened for surface T4 or T8 expression using the <sup>sitk</sup> 50% rosetting procedure or flow cytometry.

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5 -2 culture supernatants were used to infect mouse T-AM cells as described above. T4<sup>+</sup> or T8<sup>+</sup> adherent transformants were purified by the *in situ* rosetting assay followed by colony isolation; T4<sup>+</sup> or T8<sup>+</sup> non-adherent transformants were purified by fluorescence-activated cell sorting (FACS). Non-adherent human lymphoid cell lines (HSB2, RPMI-T cells; Raji - B cells) and adherent epithelial cells (HeLa) were  
10 infected by co-cultivation with T4<sup>+</sup> or T8<sup>+</sup> T-AM clones (pretreated with 10 micrograms/ml mitomycin-C for 2 hours; Sigma) and were purified.

15 Cell lines were selected for G418 resistance at a concentration of 1.5 mg/ml, except for HeLa cells which require 1 mg/ml, and fibroblasts which require 0.5 mg/ml. All cell cultures producing recombinant amphotrophic viruses (T-AM) were maintained under P3 containment conditions.

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#### AIDS Virus

25 The prototype LAV strain of HTLV-III/LAV was obtained from J.-C. Cherman (Institut Pasteur, Paris; (45)). Virus inocula used in these studies were from the second to fifth passages of virus in our laboratory. Inocula are culture supernatants from HTLV-III/LAV-infected, phytohemagglutinin (PHA)-stimulated peripheral lymphocytes which were harvested by sequential centrifugation (300 x g for 7 minutes followed by 1500 x g for 20 minutes), and were stored in liquid nitrogen. For binding studies, virus was concentrated from culture supernatants, harvested as above, by ultracentrifugation at 90,000 x g for 90  
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minutes over a 15% cushion of Renograffin (E.R. Squibb) in 0.01 M Tris, 0.15 M NaCl, 1 mM EDTA, pH 8.0.

Anti-HTLV-III/LAV Reagents

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Serum with high levels of antibody to HTLV-III/LAV was obtained from a homosexual man with chronic lymphadenopathy, and its specificity by immunofluorescence (46), Western blot analysis (47), and radioimmuno-  
10 precipitation (48) has been described. Portions of the IgG fraction were coupled with fluorescein isothiocyanate (FITC; FITC:protein ratio of 10.7 micrograms/ml), horseradish peroxidase (HPC; type VI; Sigma) and agarose as described (47, 49, 50, 51). Conjugates of IgG from a nonimmune serum were prepared  
15 in parallel.

Reverse Transcriptase Assay

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Magnesium-dependent, particulate reverse transcriptase (RT) activity was measured with a template primer of  $(A)_n(dT)_{12-18}$  (or  $(dA)_n(dT)_{12-18}$  as the negative control) in the presence of 7.5 mM  $Mg^{2+}$  (52).

25

Immunofluorescence Detection of Cytoplasmic AIDS Virus

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Cultured cells ( $1 \times 10^5$  in 0.1 ml) were centrifuged onto glass slides (Shandon Cytocentrifuge), fixed in 95% ethanol and 5% acetic acid at -20°C for 30 minutes, and rehydrated with three 10 minute changes of PBS (0.01 M  $PO_4$ , 0.15 M NaCl, pH 8.0). Slides were exposed to a 1/500 dilution of FITC-anti-HTLV-III/LAV (19 micrograms/ml) for 30 minutes at room temperature. The slides were then washed (three changes, 10 minutes each) and mounted under a coverslip with 50% glycerol  
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in PBS. The slides were examined with an epi-illuminated Leitz Orthoplan microscope at 630 x power. Under these conditions, the FITC-anti-HTLV-III/LAV reagent is specific for HTLV-III/LAV. Uninfected PHA-stimulated cells, Epstein Barr (EB) virus-infected B cell lines, an adenovirus-infected cell line, several T cell lines, and HTLV-I and HTLV-II infected cell lines were not stained.

10      AIDS Virus Immunoassay (Antigen Capture Assay)

This is a sandwich immunoassay that has been described in detail (47). Briefly, culture supernatant is added to microtiter plate wells coated with anti-HTLV-III/LAV IgG. After the plates are washed, bound virus antigen is detected with HPO-anti-HTLV-III/LAV. This assay, which is at least as sensitive as the RT assay, is negative with culture supernatants from PHA-stimulated lymphocytes from numerous donors, EB virus-infected B cell lines, several T cell lines, polyclonal and cloned IL-2 dependent T cell lines, the myeloid line K562, as well as cell lines that harbor HTLV-I or HTLV-II. The cutoff CD<sub>490</sub> for discriminating a positive from a negative supernatant was determined in each run from the mean plus 2 SD of at least 10 replicative determinations on control (uninfected cell culture) supernatants harvested at the same time.

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AIDS Virus Infectivity (ID-50) Assay

The microculture assay for the titration of infectious HTLV-III/LAV has been described in detail (47). Briefly, PHA-stimulated lymphocytes or cell lines ( $2 \times 10^6$  cells/ml) are inoculated with serial 10-fold dilutions of virus inoculum and incubated for 18 hours at 37°C. The cells were then washed and plated in microculture (10 to 20 cultures per dilution:  $1 \times 10^5$  cells per culture in 0.25 ml medium). Every 4 days, 100 microliters of supernatant was removed and replaced with fresh medium. Supernatants were then assayed for viral antigen by the antigen capture assay as described above. Infectious virus titer (ID-50) is defined as the reciprocal of the dilution at which 50% of the cultures are positive for virus (47).

VSV Pseudotype Assay

Vesicular stomatitis virus (VSV, Indiana strain, wild type) was propagated in cells producing the retrovirus required for the envelope pseudotype as described (53). Hyperimmune neutralizing sheep anti-VSV serum was added to the harvested VSV to inactivate non-pseudotype virions. The pseudotype titers ranged between  $10^4$  and  $10^5$  PFU/ml. For the assay,  $2 \times 10^5$  cells to be infected with VSV pseudotypes were plated in 30-mm diameter tissue culture wells. HeLa, NIH 3T3, and L cells were naturally adherent; all other cells types were attached by pretreatment of the substratum with 50 micrograms/ml poly-L-lysine. After virus adsorption for 1 hour, the cells were washed and  $10^6$  mink CCL64 or bovine MDBK cells were added to each well. These cells provide excellent plaques for secondary VSV infection but are resistant to infection by pseudotype

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5 virions. After allowing the plaque indicator cells to settle and spread (approximately 90 minutes), the monolayers were overlaid with agar medium. VSV plaques were counted 2 days after infection. Anti-T4A monoclonal antibody (1:20), anti-HTLV-III serum (1:10), or anti-HTLV-I serum (1:10) were used to inhibit pseudotype plaque formation by pretreatment of cells 30 minutes before addition of pseudotypes as described by (54).  
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#### Syncytium Induction Assay.

15 2 x 10<sup>5</sup> cells were co-cultivated with 2 x 10<sup>4</sup> H9 cells infected by and producing HTLV-III (55) in 10 mm diameter wells. The cultures were incubated at 37°C and examined for syncytia formation after 18 hours as previously described (54, 56). Cells were five or more syncytia were scored as positive. Syncytium inhibition was assayed by adding anti-T4A monoclonal antibody (1:20) to the mixed cultures at the time of seeding.  
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#### Cytofluorometric Analysis and AIDS Virus Binding

25 The method has been described in detail (46). Briefly, cell surface T4 or T8 expression was detected by direct immunofluorescence with fluorescein-conjugated anti-T4A or anti-T8 monoclonal antibodies (CKT\*4A, OKT\*8). The diluent/wash buffer was 0.01 M PO<sub>4</sub>, 0.15 M NaCl, pH 7.4, containing 0.1% bovine serum albumin, 2% v/v AB<sup>+</sup> human serum, and 0.01% NaN<sub>3</sub>. All reagents were pretitered for optimal (saturating) binding. Cells (5 x 10<sup>5</sup>) were incubated in a 25 microliter dilution of monoclonal antibody for 30 minutes at 4°C. The cells were washed by centrifugation (300 x g for 7 minutes), resuspended in 0.5 ml of 1% paraformaldehyde in saline,  
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and analyzed with a fluorescence-activated cell sorter (FACS IV, Becton Dickinson). For HTLV-III/LAV binding, 5 x 10<sup>5</sup> cells were incubated with HTLV-III/LAV (500 ng in 10 microliters) for 30 minutes at 37°C. Washed cells were resuspended in 25 microliters of fluorescein-conjugated anti-HTLV-III/LAV for 30 minutes at 4°C. The cells were washed, resuspended in 1% paraformaldehyde, and analyzed by FACS as above. For inhibition of HTLV-III/LAV binding, cells were preincubated with anti-T4A or anti-T8 (20 ng in 20 microliters) for 30 minutes at 4°C followed by addition of HTLV-III/LAV (500 ng in 10 microliters) for 30 minutes at 37°C. The cells were washed, incubated with fluorescein-conjugated anti-HTLV-III/LAV, washed, resuspended in paraformaldehyde, and analyzed by FACS as above.

Cell Surface Radioiodination, Immunoprecipitation, and Gel Electrophoresis

T4<sup>+</sup> NIH 3T3 transformants were surface radioiodinated by the lactoperoxidase technique (18) as follows: 4 x 10<sup>7</sup> cells were suspended in 1 ml of PBS containing 0.5 mM EDTA, 2 mCi Na<sup>125</sup>I, and 20 micrograms lactoperoxidase. At times 0, 1, 5, 10, and 15 minutes, 10 microliters of 0.03% H<sub>2</sub>C<sub>2</sub> were added. The reaction was carried out at 23°C and was stopped at 20 minutes by 2 centrifugations in 50 volumes of cold PBS containing 10 mM NaI. Labeled cells were split into 4 tubes and incubated, as indicated, with HTLV-III/LAV (2 micrograms in 20 microliters) for 30 minutes at 37°C. Subsequent washes and manipulations were performed at 0° to 4°C. Washed cells were lysed by adding 1 ml of detergent lysing buffer (LB; 0.02 M Tris, 0.12 M NaCl, pH 8.0, containing 0.2 mM phenylethylsulfonylfluoride, 5

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micrograms/ml aprotinin, 0.2 mM EGTA, 0.2 mM NaF, 0.2% sodium deoxycholate, and 0.5% (v/v) Nonidet P-40). Tubes were held on ice for 15 minutes, and nuclei were removed by centrifugation at 3000 x g for 20 minutes.

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For absorptions, Sepharose conjugates of human anti-HTLV-III/LAV IgG, human nonimmune IgG, anti-T4A, and anti-T8 antibodies were prepared as described (48). Lysates were preabsorbed with 200 microliters of Sepharose-nonimmune human IgG for 1.5 hours with rotation, and then immunoprecipitated with 20 microliters of Sepharose conjugates (as indicated) for 3 hours with rotation. Sepharose absorbants were washed 3 times: once with LB; once with LB containing 0.5 M NaCl; and once with LB containing 0.1% sodium dodecyl sulfate (SDS). Absorbed material was eluted at 65°C for 30 minutes with 20 microliters of sample buffer (0.01 M Tris, pH 8.0, containing 2% SDS, 5% 2-mercapto-ethanol (v/v), 25 micrograms bromphenol blue, and 10% glycerol (v/v)). Electrophoresis was performed in a 3.3-20% gradient polyacrylamide gel with a 3% stacking gel (57), and autoradiographs were developed with Kodak XAR-5 film.

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### Virus Inhibition Assay

2 x 10<sup>5</sup> T4<sup>+</sup> JM T cells were exposed to AIDS virus at 0 minutes. The inhibitors ammonium chloride (20 mM) or amantadine (20 mM) were added at various times during the course of virus infection (0 minutes, 30 minutes, and 60 minutes). After 6 hours, cells were washed and replated in fresh medium (RPMI/10%FCS). The effect of these agents on AIDS virus infection was determined 5 days post infection. The fraction of infected cells in the cultures expressing viral antigens was determined

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by immunofluorescence microscopy as described above (58).

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RNA Isolation and Northern Blot Hybridizations

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Total RNA was isolated from cells by homogenation in 4M guanidinium thiocyanate, followed by ultracentrifugation through a 5.7 M CsCl cushion (28). Poly(A)<sup>+</sup> selection was achieved by oligo(dT)-cellulose chromatography (Type 3, Collaborative Research) (29).

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RNA was electrophoresed through a 1% agarose-formaldehyde gel (39) and transferred onto Hybond (Amersham). Northern blot hybridization was performed according to the procedures supplied by the manufacturer. Probes were nick-translated to a specific activity of 0.5-1  $\times$  10<sup>9</sup> cpm/microgram with  $\alpha^{32}P$ -labeled deoxynucleotide triphosphates (59).

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RESULTSIsolation of a T4 cDNA

5      The strategy used to isolate a T4 cDNA initially involved constructing L cell transformants that express T4 on their surface. cDNA synthesized from the mRNA of a T4<sup>+</sup> transformed fibroblast was enriched by subtractive hybridization and used as a probe to isolate a cDNA encoding T4 from a cDNA library made from the mRNA of peripheral T lymphocytes. The identity of T4<sup>+</sup> cDNA clones was determined by Northern and Southern blot analyses, and ultimately by the ability of these clones to transfer the T4<sup>+</sup> phenotype to recipient cells.

10     Similar techniques have previously been employed to isolate the gene encoding the T8 protein (20).

20     Mouse L cells deficient in thymidine kinase (tk) were cotransformed with genomic DNA from the T cell leukemic cell line HUT-102 along with the tk-containing plasmid, pTK (25, 26). tk<sup>+</sup> L cell transformants expressing T cell surface proteins were identified by an *in situ* rosetting assay. tk<sup>+</sup> colonies were exposed to mouse monoclonal antibodies directed against T4 and were then incubated with red blood cells coupled with rabbit anti-mouse immunoglobulin. T4<sup>+</sup> transformants are visibly red by virtue of their specific association with red blood cells. In this manner, one primary T4<sup>+</sup> transformant, LTD-4, was obtained. The expression of the T4 molecule by this clone was independently verified by cytofluorometric analysis (Figure 1).

35     The mRNA population of the T4<sup>+</sup> transformant, LTD-4, should differ from that of an untransformed L cell only

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in the expression of newly transformed genes. These sequences were enriched for by annealing highly radioactive cDNA prepared from poly(A)<sup>+</sup> RNA of the T4<sup>+</sup> transformant with a vast excess of RNA from an untransformed L cell (32, 60). cDNA incapable of hybridizing, even at high Rot values, was isolated by hydroxyapatite chromatography and used to screen a human peripheral T cell cDNA library constructed in the lambda cloning vector gt10. Four weakly hybridizing plaques were identified, plaque-purified and analyzed for the presence of T4 sequences.

To determine whether any of these clones encoded T4, Northern blot analyses were initially performed with RNA from T4<sup>+</sup> and T4<sup>-</sup> peripheral T cells, leukemias, thymocytes, L cell transformants and nonlymphoid cells (Figure 2). One of the four clones hybridized to an RNA present only in T4<sup>+</sup> cells. This clone detects a 3 kb RNA present in the T4<sup>+</sup> transformant, LTD-4, which is also present in a population of T4<sup>+</sup> peripheral lymphocytes, a variety of T4<sup>+</sup> leukemic cell lines, and thymocytes. No hybridization was observed with RNA from untransformed fibroblasts, T4<sup>-</sup> peripheral lymphocytes, HeLa cells, or human neuroblastoma cells.

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The pattern of expression of RNA detected by this clone is consistent with the possibility that it encodes T4. However, this cDNA is only 0.6 kb in length but hybridizes to a 3 kb mRNA. Therefore, the human peripheral T cell cDNA library was rescreened and one clone (pT4B) was obtained which contained a 3 kb insert, close in size to that of the mature messenger RNA. Restriction maps of this clone are shown in Figures 3A and 3B.

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Genomic Blot Analysis

Southern blot experiments (37) were next performed to demonstrate that the isolated cDNA clone hybridized with DNA from the T4<sup>+</sup> transformant as well as human DNA, but not with untransformed mouse L cell DNA (Figure 4). Genomic DNA from a variety of human cells reveals a set of five hybridizing fragments after cleavage with the enzyme BamHI. As expected, T4 sequences can be detected in the transformant LTD-4, but not in untransformed L cell DNA. The BamHI fragment closest to the 3' end of the gene (6.6 kb) is not present in LTD-4, presumably as a consequence of the integration event. Moreover, no gross rearrangements are apparent at this coarse level of analysis when comparing DNA from lymphoid and nonlymphoid cells. The sum of the molecular weights of the hybridizing fragments is 33 kb, suggesting that the T4 gene is quite large. A complete set of genomic clones spanning this region was obtained (see below) and the BamHI fragments were ordered by restriction analysis of these clones (Figure 3A), confirming that the gene is large and must contain introns of significant lengths.

25 Expression of the T4 cDNA in Transformed Mouse Fibro-  
blasts

Further evidence that the isolated cDNA encodes T4 would be provided if this clone could convert fibroblasts to the T4<sup>+</sup> phenotype after transformation. The T4 gene in chromosomal DNA is large and spans several genomic clones. Therefore, the cDNA clone was introduced into two retroviral expression vectors, pVcos7 and pMV6kt/neo, which contain the Moloney murine leukemia virus long terminal repeats (LTRs) flanking a sin-

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gle EcoRI cloning site (Figure 3C). The 5'-LTR promotes transcription through the cloning site and the 3'-LTR contains sequences necessary for cleavage and polyadenylation. The vector pMV6tk/neo also contains the tk promoter fused to the coding region of the neomycin phosphotransferase gene. The construct employing pVcos7 requires transformation with an unlinked selectable marker, whereas pMV6tk/neo carries the neomycin resistance marker, which permits linked cotransformation. Neo<sup>r</sup> colonies of NIH 3T3 cells obtained after transformation were selected by their ability to grow in media containing the neomycin analogue G418, and were screened using the rosetting procedure to detect the expression of T4 on the cell surface. Approximately 50% of the G418 colonies obtained with pVcos7 and 75% of the colonies obtained with pMV6tk/neo were positive for T4 in this assay. Rosette-positive colonies were further analyzed by cytofluorometry to confirm that T4 is expressed on the transformed cell surface (Figure 1).

Metabolic protein labeling experiments were performed which demonstrate that the T4<sup>r</sup> transformed fibroblast and the T lymphocyte express a T4 protein of identical molecular weight. Untransformed NIH 3T3 cells, T4<sup>r</sup> transformants and T lymphocytes were labeled for 12 hours in the presence of L-[<sup>35</sup>S]-methionine (41). The cells were detergent solubilized and the lysate was passed over lentil lectin columns to enrich for glycoproteins (42). The bound glycoprotein fraction was eluted and immunoprecipitated with monoclonal antibodies directed against T4 (Figure 5). Under reducing conditions, a glycoprotein migrating at a relative molecular mass of 55 kd is detected in extracts from T lymphocytes and two independent T4<sup>r</sup> transformants.

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This protein is not detected in control 3T3 fibroblasts. Under nonreducing conditions, a 51 kd glycoprotein is immunoprecipitated with anti-T4 in T cells and in the transformed fibroblasts.

These experiments demonstrate that the transformants express a 55 kd glycoprotein immunoprecipitated with anti-T4 which is identical in size to that expressed on the surface of T lymphocytes. Thus, Northern and Southern analyses using the isolated cDNA, taken together with the ability of this cDNA to confer the T4<sup>+</sup> phenotype to mouse fibroblasts, indicate that the entire coding sequence of the T cell surface protein T4 had been cloned.

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Nucleotide Sequence of the T4 cDNA and the Deduced Protein Sequence

The complete nucleotide sequence of the T4 coding region was determined by sequencing both strands of the 3 kb cDNA insert using the dideoxy termination method (35, 36). The complete nucleotide sequence and the predicted protein sequence are shown in Figure 6. The longest open reading frame begins at position 76 with a methionine codon surrounded by the initiation consensus sequence PurNNATGPur (61). This reading frame extends 1374 nucleotides, encoding a polypeptide containing 458 amino acids. The contiguity of this reading frame was confirmed by inserting this cDNA into the RNA expression vector pSP6 (40). RNA synthesized from this vector, when translated *in vitro*, directs the synthesis of an unmodified 51 kd protein, the precise molecular weight predicted from the nucleotide sequence (Figure 7).

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T4 is comprised of a leader sequence, four tandem variable-joining (VJ)-like regions, and a membrane-spanning domain each sharing homology with corresponding regions of different members of the immunoglobulin gene family (62, 63) (Figures 6 and 8). A stretch of hydrophobic residues, corresponding to a leader peptide predicted by a Kyte-Dolittle (64) hydropathicity plot, immediately follows the initiation codon. Although the exact position at which the native T4 protein is processed cannot be determined, it is contemplated that cleavage occurs just after the threonine at positions -1 based on known cleavage patterns (65). Therefore, the signal peptide contains 23 amino acids and the processed T4 protein consists of 435 residues.

Residues 1-94 of the mature protein share both amino acid and structural homology with the immunoglobulin light chain variable domain (Figure 9A). The overall homology of this domain with immunoglobulin variable regions is 32%. Sequence comparison between the V regions of light chain immunoglobulins and the N-terminal V-like region (V1) of T4 demonstrates that eight out of 14 invariant residues are conserved (66). This domain contains two cysteine residues, separated by 67 amino acids, whose positions and spacing are analogous to that found in light chain immunoglobulins and related molecules (67). These cysteines may be capable of forming the conserved intrastrand disulphide bond characteristic of V domains. This suggestion is supported by our observation that T4 migrates more rapidly under nonreducing conditions than under reducing conditions, consistent with the formation of at least one intrastrand linkage (Figure 5, lanes e and f).

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Aside from homologies at the level of individual amino acids, the V1 domain of T4 shares structural features with immunoglobulin variable regions. Immunoglobulin variable and constant domains fold in a characteristic pattern in which a series of antiparallel  $\beta$ -strands fold to form two  $\beta$ -sheets (67, 68). These  $\beta$ -sheets are held together both by a disulphide bridge and by characteristic hydrophobic interactions. To determine how the predicted secondary structure of the V-like domain of T4 compares with the structure of the V domains of light chain immunoglobulins, two-dimensional structural alignments were performed. Also, a plot of probable  $\beta$ -strands and  $\beta$ -turns in these sequences using the empirically derived algorithm of Chou and Fasman (69) was obtained. These analyses suggest the presence of seven  $\beta$ -strands within the V-like domain of T4 which closely match those found in the immunoglobulin V domain (Figure 9A). The two conserved cysteines of T4 are found within  $\beta$ -strands B and F, matching exactly the positions of the cysteines in the V region known to form the conserved disulphide bond in immunoglobulin. A tryptophan residue lies 12 amino acids downstream of the first cysteine and a tyrosine residue is situated two amino acids before the second cysteine. These residues are highly characteristic of  $\beta$ -strands C and F, respectively in light chain V regions. In addition, an aspartate residue is found six amino acids before the second cysteine, and an arginine residue lies at the base of  $\beta$ -strand D. These charged residues are highly characteristic of V domains (67). Finally, patches of alternating hydrophobic residues are present throughout the  $\beta$ -strands, which strengthen the interaction of the two  $\beta$ -sheets.

The V1 domain of T4 is followed by a stretch of amino acid residues bearing significant homology to the joining (J) regions of immunoglobulins and T cell antigen receptors. In Figure 9B, this J-like region of T4 is aligned with the consensus joining sequences of immunoglobulin light chains and the two chains of the T cell antigen receptor. This J-like region is followed by a 265 amino acid stretch which may be structurally divided into three additional VJ-like domains with statistically significant sequence and structural homology to prototype immunoglobulin VJ regions (Figures 6 and 8). Additionally, this sequence contains two potential N-linked glycosylation sites (Asn-Leu-Thr; Figure 6).

The extracellular domain is followed by a putative transmembrane sequence, predicted by a hydropathicity plot (64), which contains only hydrophobic and neutral amino acid residues. This segment bears striking homology to the transmembrane exon of the  $\beta$ -chains of class II major histocompatibility proteins (Figure 9C). Alignment of the transmembrane regions of T4 and MHC class II  $\beta$ -chains reveals 48% homology without gaps. Following the membrane-spanning segment, a highly charged sequence of 40 amino acids comprise the cytoplasmic domain (Figures 6 and 8).

The T4 Gene: Chromosomal Location and Intron-Exon Positions

The T4 cDNA was used to determine the chromosomal location of the T4 gene by analyzing its segregation pattern in a panel of mouse-human somatic cell hybrids and by *in situ* hybridization to human metaphase chromosomes (101). Genomic blot experiments and in

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*situ* hybridization indicate that the T4 gene resides on the short arm of human chromosome 12, between regions 12p12 and 12pter.

5 A set of overlapping genomic clones spanning the T4 gene was obtained by screening human genomic libraries constructed in the lambda cloning vectors Charon 4 and EMLB-3 (31) with a radiolabeled pT4B cDNA insert (70). Characterization of these clones by both restriction 10 and Southern blot analyses indicated that they contained the entire T4 coding sequence. The complete intron-exon organization of the T4 gene was then determined by sequencing specific fragments of the genomic clones using the dideoxy termination procedure 15 (35, 36).

20 The T4 gene is comprised of 9 exons split by 8 introns as shown in Figures 8 and 10. The first exon contains the 5'-untranslated region and the leader segment. The first variable-like domain, V<sub>1</sub>, is split by a large 25 intron located at nucleotide position 289 (Figure 6). Therefore, the V<sub>1</sub>J<sub>1</sub> domain is encoded by the second and third exons and the V<sub>2</sub>J<sub>2</sub>, V<sub>3</sub>J<sub>3</sub>, V<sub>4</sub>J<sub>4</sub>, and transmembrane (TM) domains are each encoded by separate exons (exons 4-7). The cytoplasmic domain (CYT) is split by an 30 intron and the last portion of the cytoplasmic domain and the 3'-untranslated region are encoded by the ninth exon.

30 The Construction of T4<sup>+</sup> and T8<sup>+</sup> Transformed Cells

35 The experimental approach used to study the role of T4 in AIDS virus infection initially involved the introduction of the T4 gene into T4<sup>-</sup> cell lines incapable of supporting viral infection. The

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transformed cells were then tested for susceptibility to AIDS virus, followed by studies on the mechanism by which T4 mediates viral infection.

5       A full length cDNA clone encoding the surface protein T4 was subcloned into the retroviral expression vector, pMV7. The expression vector, pMV7 (Figure 11A), contains two directly repeated Moloney murine sarcoma virus long terminal repeats (LTRs) which flank a single  
10      EcoRI cloning site. The 5'-LTR constitutively promotes transcription through the cloning site, whereas the 3'-LTR provides sequences necessary for cleavage and polyadenylation of the RNA. In addition, pMV7 contains the herpesvirus thymidine kinase promoter (tk) fused to  
15      the coding region of the bacterial neomycin phosphotransferase gene (neo), a dominant selectable marker, permitting linked cotransformation and infection.

20      T4-pMV7 was introduced into  $\gamma$ -2 and  $\gamma$ -AM cells, NIH 3T3 cell lines containing defective ecotropic and amphotropic proviruses, respectively (Figure 11B) (44,59). Both cell lines are incapable of encapsidating endogenous viral RNA but can provide all obligate trans  
25      viral functions. Stable transfection of these cell lines with T4-pMV7 results in the production of recombinant retroviral stocks encoding T4 which are free of helper virus. These pure viral stocks can then be used to efficiently introduce T4 sequences into both mouse and human cells without the production of retrovirus by the target cell.

30      Briefly, T4-pMV7 DNA was introduced into  $\gamma$ -2 cells using the procedure of DNA-mediated gene transfer (Figure 11B) (25, 27). Neo<sup>+</sup> positive colonies were

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selected by their ability to grow in media containing the neomycin analog G418 (Geneticin<sup>®</sup>) and screened for the expression of T4 on the cell surface using an *in situ* rosetting assay (20, 70). Colonies of transfected  $\gamma$ -2 cells expressing T4 were then identified which produce recombinant retrovirus in titers of  $10^5$  cfu/ml. T4<sup>+</sup>  $\gamma$ -2 clones were then used to generate retroviruses capable of infecting mouse  $\gamma$ -AM cells. T4 expressing  $\gamma$ -AM clones were isolated which yield recombinant retroviral titers of  $10^4$  cfu/ml. T4<sup>+</sup> human transformants were generated by co-cultivation of cells with mitomycin-C treated or  $\gamma$ -AM clones (Figure 11B). T4<sup>+</sup> transformants were subsequently analyzed by Northern blot analysis and flow cytometry to confirm that T4 is expressed and is present on the cell surface. Control cell lines expressing the surface protein T8 were constructed in an analogous manner.

#### T4 is Essential for AIDS Virus Infection

To initially determine whether the presence of the T4 protein on the surface of a human lymphocyte is sufficient to render the cell susceptible to AIDS virus infection, transformants of the primitive T cell leukemic line, HSB2 (71), which expresses only the early T lymphocyte proteins T1 and T11 on its surface, were constructed. HSB2 expresses neither T4 nor T8, nor does it express the T cell antigen receptor or the associated complex of T3 proteins. Transformants of HSB2 which express either the T4 or T8 proteins on the cell surface were selected and used to determine the susceptibility of these cell lines to AIDS virus infection. Several different experimental approaches were employed to assess AIDS virus infection, including expression of reverse transcriptase (52), expression of

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virus in the cytoplasm of the cell by immuno-fluorescence microscopy (46), detection of viral antigens in the culture supernatant using an immunoassay (47), as well as production of infectious virions by supernate subculture with phytohemagglutinin (PHA)-stimulated peripheral lymphocytes (46). Using these assays, evidence of AIDS virus infection of the HSB2 cell line was not observed (Table I).

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Table 1Susceptibility of T4<sup>+</sup> and T8<sup>+</sup> Human Transforms to AIDS Virus Infection

<u>HUMAN CELL</u>	<u>Maximum Reverse Transcriptase</u>	<u>Cytoplasmic Virus</u>	<u>Supernate Viral Ag</u>	<u>Supernate Subculture</u>	<u>Syncytium Induction</u>	<u>VSV(AIDS) Pseudotype Infection</u>	<u>Virus Binding</u>
CEM(T4 <sup>+</sup> )	675023	+	+	+	+	+	+
IS82	4245	-	-	-	-	-	-
IS82-T8 <sup>+</sup>	4460	-	-	-	-	-	-
IS82-T4 <sup>+</sup>	190915	+	+	+	+	+	+
Raji	ND	ND	ND	ND	-	-	ND
Raji-T8 <sup>+</sup>	5595	-	-	-	-	-	-
Raji-T4 <sup>+</sup>	103500	+	+	+	+	+	-
HeLa	6438	-	-	-	-	-	-
HeLa-T8 <sup>+</sup>	4875	-	-	-	-	-	-
HeLa-T4 <sup>+</sup>	48125	+	+	+	+	+	+

$5 \times 10^6$  cells were inoculated with AIDS virus, incubated at 37°C for 24 hours, washed, and replated in fresh media. Cells and supernatants were removed at days 3, 6, 9, 12, 16, 20, 24, and 28 and used in four virus detection assays: reverse transcriptase, cytoplasmic virus, supernate viral antigen, and supernate subculture.

The results of the pseudotype infection experiments are expressed as follows: + ( $\geq 10^3$  PFU/ml); - (10 PFU/ml); ND, not determined.

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5 In addition, it has been previously demonstrated that extensive cell fusion occurs when uninfected human  
cells bearing receptors for AIDS virus are co-  
cultivated with cells producing AIDS virus (54). In  
this assay, there is no induction of syncytia when HSB2  
cells are mixed with AIDS virus-producing H9 cells  
10 (Table I), although abundant syncytia are formed with  
HTLV-I and HTLV-II producing cells (data not shown).

15 Finally, viral entry was tested for using pseudotypes  
of vesicular stomatitis virus (VSV) bearing the  
envelope glycoproteins of the AIDS virus (Table I)  
(53, 54). When cells infected with AIDS virus are  
superinfected with VSV, a proportion of the progeny VSV  
20 assemble sufficient AIDS virus envelope glycoprotein to  
resist neutralization by hyperimmune anti-VSV serum.  
The host range of these VSV (AIDS) pseudotype virions  
is restricted to cells expressing receptors specific to  
the AIDS virus. Following penetration of the cell and  
uncoating of the virion, the transcapsidated VSV genome  
replicates to produce non-pseudotype particles. During  
25 the secondary infection, progeny VSV released from  
infected cells penetrate and destroy neighboring  
indicator cells resistant to VSV (AIDS) pseudotype  
infection (mink CCL64 or bovine MDBK cells), resulting  
in the formation of VSV plaques which are then scored.  
Thus, infection with VSV (AIDS) pseudotypes provides a  
30 quantitative cytopathic plaque assay for viral entry  
(54). In this assay, no plaques over background were  
observed when HSB2 cells were exposed to VSV (AIDS)  
pseudotypes (Table I). In control experiments with  
35 pseudotypes of VSV RNA encapsidated in an HTLV-I  
envelope (VSV (HTLV-I)), numerous plaques were  
observed, demonstrating that the HSB2 cell, which bears

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5        HTLV-I receptors, is capable of replicating VSV efficiently. These observations demonstrate that the VSV genome encapsidated in an AIDS virus envelope is incapable of entering HSB2 cells.

10      Whether the introduction of a functional T4 cDNA into HSB2 would render this cell susceptible to AIDS virus infection was next studied (Table I). Exposure of HSB2-T4<sup>+</sup> transformants to AIDS virus results in a productive viral infection as determined by expression of reverse transcriptase activity (52), expression of virus in the cytoplasm of the cell by immunofluorescence microscopy (46), detection of viral antigen in the culture supernatant using an immunoassay (47), as well as the production of infectious virus by supernate subculture with PHA-stimulated lymphocytes (Table I) (46). Control HSB2-T8<sup>+</sup> cells were consistently negative in each of the assays.

20      In addition, the efficiency with which different T4<sup>+</sup> T cells are infected with AIDS virus was also examined. HSB2-T4<sup>+</sup> and HSB2-T8<sup>+</sup> transformants, the naturally-isolated T4<sup>+</sup> T cell line CEM, as well as PHA-stimulated peripheral lymphocytes were exposed to serial 10-fold dilutions of AIDS virus, washed, and plated in microculture. The frequency of infected cultures was then determined using an immunoassay 12 days after exposure to virus (Figure 12) (47). In this manner, the titer of AIDS virus required to infect 50% of the exposed cultures (ID-50) was defined. The ID-50 of PHA-stimulated peripheral lymphocytes is 2-3 orders of magnitude greater than that observed for either naturally-isolated or transformed T4<sup>+</sup> cell lines. The efficiency of infection of HSB2-T4<sup>+</sup> cells is about 10 fold higher than that observed for the naturally-

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isolated T4<sup>+</sup> T cell line CEM (Figure 12). Control HSB2-T8<sup>+</sup> cells are not susceptible to infection even at the highest virus titers examined.

5      The ability of HSB2-T4<sup>+</sup> cells to support both syncytia formation and the replication of VSV (AIDS) pseudotypes was also studied. When HSB2-T4<sup>+</sup> cells are co-cultivated with AIDS virus producing H9 cells, syncytia formation is readily observed within 18 hours (Tables I and II). Moreover, syncytium induction is abolished by pretreating cultures with anti-T4A monoclonal antibody (Table III). Finally, when HSB2-T4<sup>+</sup> cells are exposed to VSV (AIDS) pseudotypes, infectious VSV particles are produced which destroy neighboring indicator cells (Tables I and III). Furthermore, plaque formation is inhibited by pretreatment with either anti-AIDS virus antibody or anti-T4A monoclonal antibody (Table III). Control HSB2-T8<sup>+</sup> cells are consistently negative in each of the seven assays employed to detect AIDS virus infection (Tables I, II, and III). These observations provide genetic evidence that in an immature human T lymphocyte, the mere presence of the T4 protein provides an essential function required for AIDS virus infection.

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Table II  
Induction of Syncytia in T4<sup>+</sup> Human Transformants

<u>HUMAN CELLS</u>	<u>SYNCYTIA INDUCTION</u>	
	<u>H9/AIDS</u>	<u>H9/AIDS + aT4A</u>
JM (T4 <sup>-</sup> )	----	-
8166 (T4 <sup>+</sup> )	----	-
HSB2	-	ND
HSB2-T8 <sup>+</sup>	-	ND
HSB2-T4 <sup>+</sup>	--	-
Raji	-	ND
Raji-T8 <sup>+</sup>	-	ND
Raji-T4 <sup>+</sup>	---	-
HeLa	-	ND
HeLa-T8 <sup>+</sup>	-	ND
HeLa-T4 <sup>+</sup>	----	-

2 x 10<sup>5</sup> cells were co-cultivated with 2 x 10<sup>4</sup> AIDS virus-producing H9 cells (H9/AIDS) and incubated at 37°C. The cultures were examined for syncytia formation after 18 hours. The results are expressed as the approximate percentage of nuclei contained within syncytia: - (no syncytia); ++ (25%); +++; (50%) ++++(90%); ND (not determined). Syncytium inhibition was assayed by adding anti-T4A monoclonal antibody (aT4A; 1:20) to the mixed cultures at the time of seeding. The naturally-isolated T4<sup>+</sup> T cell lines JM and 8166 served as positive controls in these studies.

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Table III

VSV Pseudotype Cytopathic Plaque Assay on T4<sup>+</sup> and T8<sup>+</sup> Human Transformants

<u>HUMAN CELLS</u>	<u>VSV PSEUDOTYPE TITER (PFU/ml)</u>		
	<u>VSV(HTLV-I)</u>	<u>- HTLV-I</u>	<u>VSV(AIDS)</u>
		<u>- AIDS</u>	<u>- T4A</u>
CEM(T4 <sup>+</sup> )	20,000	50	42,000
HSB2-T8 <sup>+</sup>	10,000	50	0
HSB2-T4 <sup>+</sup>	12,000	50	1,000
Raji-T8 <sup>+</sup>	5,000	ND	0
Raji-T4 <sup>+</sup>	5,000	50	1,500
HeLa	10,000	ND	0
HeLa-T4 <sup>+</sup>	10,000	50	17,000

$2 \times 10^5$  cells were incubated with VSV (AIDS) pseudotypes (53, 54) for 1 hour at 37°C. The cells were then washed and  $1 \times 10^6$  mink CCL64 or bovine MDBK plaque indicator cells, permissive to VSV infection but resistant to VSV (AIDS), were added to each well. The cultures were then overlaid with agar medium and scored for VSV plaques two days post infection. Anti-T4A monoclonal antibody ( $\geq$ T4A; 1:20) or anti-AIDS virus serum ( $\geq$ AIDS; 1:10) were used to inhibit VSV (AIDS) pseudotype plaque formation by pretreatment of cells 30 minutes before exposure to pseudotypes (54). VSV (HTLV-I) pseudotypes, which plate on a wide variety of human cell types (54), were used as controls in these experiments. Anti-HTLV-I serum (1:10) was used to block VSV (HTLV-I) pseudotype plaque formation. The results are expressed as PFU/ml; ND (not determined).

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AIDS Virus Infection Is Not Restricted to T Lymphocytes

15 A functional T4 cDNA was introduced into two human non-T cell lines: HeLa, an epithelial cell line derived from a cervical carcinoma (72), and Raji, a B lymphoblastoid cell line derived from a patient with Burkitt's lymphoma (73) (Figure 11B). Prior to retrovirus-mediated gene transfer, these cell lines do not express surface T4 protein or T4 mRNA, nor are they susceptible to AIDS virus infection (Table I). In addition, the parental cell lines do not support the induction of syncytium nor the plating of VSV (AIDS) pseudotypes (Tables I, II and III).

20 15 In contrast, T4<sup>+</sup> Raji and HeLa transformants support AIDS virus infection by all of the criteria previously described (Table I). The efficiency with which Raji-T4<sup>+</sup> cells can be infected with AIDS virus approximates that of HSB2-T4<sup>+</sup> cells and is about 10 fold higher than the efficiency of infection of the naturally-isolated T4<sup>+</sup> T cell line CEM (Figure 12). Moreover, upon co-cultivation with AIDS virus-producing H9 cells, Raji-T4<sup>+</sup> and HeLa-T4<sup>+</sup> cells support the induction of syncytia which is abolished by pretreating cultures with anti-T4A monoclonal antibody (Tables I and II; Figure 13). In addition, exposure of these cells to VSV (AIDS) pseudotypes results in the production of infectious VSV and the formation of plaques which are inhibited by pretreatment with anti-AIDS virus antibody or anti-T4A monoclonal antibody (Tables I and III). Control Raji-T8<sup>+</sup> and HeLa-T8<sup>+</sup> transformants are consistently negative in each of these assays (Tables I, II, and III).

Therefore, the introduction of a functional T4 gene into either human T lymphocytes, B lymphocytes, or epithelial cells is sufficient to render such cells susceptible to AIDS virus infection. Taken together, 5 these observations indicate that the T4<sup>+</sup> T cell tropism observed *in vivo* is a consequence of the restricted expression of the T4 molecule and not the nature of the cell type in which it is expressed.

10 AIDS Virus Binds to Surface T4 Protein

The previous experiments provide genetic evidence that T4 expression is required for AIDS virus infection but do not provide information on the role of this molecule in the viral life cycle. The observation that surface expression of T4 is necessary for AIDS virus infection suggests that T4 is the AIDS virus receptor. Cytofluorometry was therefore used to examine the binding of AIDS virus to the surfaces of T4<sup>+</sup> and T8<sup>+</sup> transformed human cells (Table I; Figure 14). HSB2, Raji, and HeLa cells, and the T4<sup>+</sup> or T8<sup>+</sup> transformants, were incubated with AIDS virus. Following viral absorption, the cells were washed, exposed to fluorescein-conjugated anti-AIDS virus antibody, and analyzed by flow cytometry. This assay indicated that the AIDS virus binds efficiently and specifically to the human transformants expressing surface T4, but not to the T4<sup>-</sup> parental cells nor to the T8<sup>+</sup> transformants (Figure 14, column B; Table I). The binding of AIDS virus to the T4<sup>+</sup> cells is abolished by preincubation with anti-T4A monoclonal antibody but not by preincubation with anti-T8 monoclonal antibody (Figure 14, column C). Moreover, when T4<sup>+</sup> transformed cells are exposed to AIDS virus, the T4 glycoprotein coprecipitates with the viral envelope glycoprotein , 20 25 30 35

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5 suggesting a direct physical association between these molecules (data not shown). These results indicate that the AIDS virus binds to the T4 molecule on the cell surface and that this binding is independent of other T cell-specific proteins since binding occurs to all T4<sup>+</sup> cell types examined.

10 Previous studies have described two distinct pathways of entry for enveloped viruses (74, 75, 76, 77). Some viruses fuse directly with the plasma membrane, whereas others are internalized by receptor-mediated endocytosis. The acidic environment of the endosome then facilitates fusion of the viral envelope with the 15 limiting membrane of the vacuole. Infection by viruses which enter cells via the endocytic pathway can be inhibited by treating cells with agents such as weak bases which deacidify the endosome (58, 78, 79, 80). In the presence of ammonium chloride, fusion is blocked 20 in the endosome but lysosomal degradation still proceeds at a reduced rate (80).

25 The effect of ammonium chloride on AIDS virus infection of the T4<sup>+</sup> T cell line JM was therefore examined. In the absence of ammonium chloride, over 50% of JM cells exposed to AIDS virus express viral antigens five days after infection as determined by immunofluorescence microscopy. If JM cells are exposed to ammonium chloride (for 6 hours) either at the time of addition of virus or within 30 minutes after the addition of virus, greater than 95% inhibition of viral infection was observed. However, if cells were treated with ammonium chloride one hour after the addition of virus, no inhibition of infection was observed, a finding 35 consistent with the kinetics of viral entry described

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for other viruses which enter cells via receptor-mediated endocytosis. Finally, the ammonium chloride effect was completely reversible. Cells exposed to ammonium chloride for one hour, and then washed free of the compound and exposed to AIDS virus, supported control levels of viral infection. These results are consistent with previous observations that upon removal of ammonium chloride, the pH of the endosome returns to the original low values within 1-2 minutes (78, 80). Similar results with amantadine, a compound which deacidifies the endosome, were obtained.

These results are consistent with a mechanism of viral entry which involves endocytosis of the T4-AIDS virus complex and low pH-induced fusion of the viral envelope with the limiting membrane of the endosome, releasing the viral nucleocapsid into the cytoplasm of the cell.

T4 mRNA is Expressed in the Brain

In addition to the disruption of the cellular immune system, AIDS is frequently accompanied by central nervous system (CNS) disorders which are thought to be the consequence of the direct infection of brain cells by the AIDS virus (81). It was therefore of interest to determine whether T4 is expressed in cells within the CNS, thereby providing an explanation for the neurotropic properties of the virus. Northern blot analyses of RNA prepared from both human and mouse brains were performed to determine whether T4 mRNA sequences are expressed in the CNS (Figure 15). Poly(A)<sup>+</sup> RNA derived from human cerebral cortex contains two distinct T4 mRNAs with molecular weights of approximately 3 and 1.8 kb (Figure 15A). The weaker 3 kb RNA is identical in size to the mRNA expressed by

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two T4<sup>+</sup> leukemic cell lines, U937 (monocytic cell line) and Jurkat (T cell line), as well as by peripheral T lymphocytes. The smaller, more abundant 1.8 kb mRNA absent from T lymphocytes could result from alternative splicing or alternative 5' or 3' termini.

A more careful analysis of the localization of T4 mRNA was performed by isolating poly(A)<sup>+</sup> RNA from specific regions of the mouse brain (Figure 15B). Hybridization with radiolabeled cDNA encoding the murine homologue of T4, L3T4, reveals an intense 2.2 kb mRNA in mouse forebrain which is absent from hindbrain samples. The 2.2 kb L3T4 mRNA is detectable in the cortex, hypothalamus, and is most abundant in the striatum, but is absent from the cerebellum, brain stem, or spinal cord (data not shown). This 2.2 kb mRNA detected in the CNS is approximately 1 kb smaller than the 3.2 kb mRNA encoding L3T4 in thymocytes (Figure 15B). These results indicate that the neurotropism displayed by the AIDS virus is likely to be the result of surface expression of the T4 molecule on brain cells. The level of mRNA detected in forebrain is about 1/30th the level in thymocytes. This may reflect low level expression by a large number of cells or higher levels of expression by a small subpopulation of cells. It is not known at present whether T4 is expressed by neurons or supporting cells. The presence of a variant transcript in the CNS, however, makes it unlikely that the T4 mRNA in brain is expressed by the rare invading T-lymphocyte.

#### Discussion

The segregation of T4 and T8 with functionally distinct subsets of T cells suggests that these molecules may be

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important in the interaction of T lymphocytes with appropriate target cells. As a first step in understanding the specific role of these proteins, cDNA clones were obtained of both the T4 and T8 molecules and their nucleotide sequences were determined (20, 70). Comparison of the deduced protein sequences of T4 and T8 indicates that these molecules share significant sequence and structural homology with immunoglobulin variable (V) domains and as members of the immunoglobulin supergene family. However, the N-terminal V-like domains of T4 and T8 are quite different: they share only 28% homology and are therefore less homologous to each other than each is to immunoglobulin light chains (Figure 9A). Moreover, the regions of maximum conservation between T4 and T8 are also the regions of strongest homology to immunoglobulin and T cell receptor V regions. Thus, the immunoglobulin-like domains of these two molecules, although structurally similar, show significant sequence divergence consistent with the hypothesis that they recognize different molecules on different subsets of target cells.

The V-like region structural homology shared by the N-terminal domains of T4 and T8 may be of particular relevance to the functions of these proteins. Virtually all members of the immunoglobulin supergene family participate in the immune response (62). Moreover, the individual members of this family show a strong tendency to associate with each other to form dimers. This association is apparent in the interaction of the heavy and light chains of immunoglobulin, the alpha and beta chains of the T cell antigen receptor,  $\beta_2$ -microglobulin and class I MHC proteins and the alpha and beta chains of class II MHC molecules. The T8

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glycoprotein forms a disulphide bond with T6, a presumed MHC-like molecule, on the surface of thymocytes (82), and exists as multimers of the 32 kd subunit on peripheral T lymphocytes (83). The presence of four V-like domains in T4 indicates that these regions associate with one another as well as with specific ligands on the surface of other cells or viruses. These specific affinities of immunoglobulin-like molecules may be essential for the recognition functions of T4 and T8.

#### Evolution of T4

In the immunoglobulin and T cell antigen receptor genes, the V and J exons are widely separated and become juxtaposed only after a somatic recombination event (62, 63). The T4 mRNA encodes four contiguous V- and J-like elements without the requirement for DNA recombination events. It is therefore possible that T4 reflects a more primitive gene that evolved before the emergence of rearrangement mechanisms. Further support for this derives from recent observations that the first V-like region of T4 (V1) is split by an intron not present in the V genes encoding either the immunoglobulins or T cell antigen receptors. Accumulating evidence suggests that it is far more likely for introns to be precisely removed during evolution than for introns to be inserted in a previously intron-free environment. Thus, T4 may represent an ancestral immunoglobulin gene which underwent duplications, divergence, and rearrangement to generate the current immunoglobulin gene family. Although functional in a far more complex immune system at present, T4 may reflect receptors operative in more primitive cellular immune responses. Primitive

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immune responses, such as those of invertebrates, do not appear to involve a diverse repertoire of receptor molecules, but in the simplest cases are restricted to a distinction between self and nonself (85, 86) and are likely to be accommodated by a "static" set of genes that do not undergo rearrangement.

Whatever the order of appearance of T4 in evolutionary time, the organization of this genes reveals an interesting example of exon shuffling. T4 consists of four V-J-like domains, a J-like region and a transmembrane segment, each sharing homology with different members of the immunoglobulin supergene family. The V- and J-like domains are homologous to the equivalent regions of both immunoglobulins and the T cell antigen receptor chains; the transmembrane domain shows considerable homology to this region in the  $\beta$ -chains of class II MHC molecules (Figure 9C). T4, therefore, consists of a collection of exons conserved in several members of the immunoglobulin supergene family which are shuffled in different ways to generate a large number of different molecules which participate in the immune response.

25 T4 is the AIDS Virus Receptor

The data provided herein suggest a mechanism of AIDS virus infection which initially involves the specific association of the AIDS virus with T4 molecules on the cell surface. This association may be demonstrated on T lymphocytes, B lymphocytes, and epithelial cells, and therefore does not require the participation of additional T cell-specific proteins. Additionally, the data provided herein indicates that the T4-AIDS virus complex is internalized via receptor-mediated

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endocytosis and the viral envelope then fuses with the limiting membrane of the endosome, releasing the nucleocapsid into the cytoplasm. Viral replication and transcription can then occur in both lymphoid and non-  
5 lymphoid cell lines. Moreover, the T4 gene is expressed in the brain as well as in lymphocytes, providing an explanation for the dual neurotropic and lymphotropic character of the AIDS virus. In this manner, a T lymphocyte surface protein important in  
10 mediating effector cell-target cell interactions has been exploited by a human retrovirus to specifically target the AIDS virus to populations of T4+ cells.

Cell surface receptors have been identified for a  
15 number of enveloped viruses and the pattern of expression of these receptors is often responsible for the host range and tropic properties of specific viruses (74, 76). Some viruses will infect only a narrow range of cell types, reflecting the expression of the viral receptor on specific populations of target cells. Rabies virus, for example, interacts with the nicotinic acetylcholine receptor (87) and infects largely skeletal muscle and neurons, whereas the Epstein-Barr virus interacts with the C3d complement  
20 receptor type 2 (88) and infects B lymphocytes. Other viruses, such as the myxoviruses, interact with ubiquitously distributed sialic acid residues on the cell surface and infect a much broader range of cell types.  
25

The restricted expression of cell surface receptors provides only one explanation for viral tropism. Some viruses will replicate only in a restricted set of differentiated cell types whereas others will only be efficiently transcribed in specific cell types. Hence,  
30 the Moloney murine leukemia virus (Mo-MuLV) induces T

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cell lymphomas in newborn mice., yet the closely-related Friend helper murine leukemia virus (Fr-MuLV) induces primarily erythroleukemias (89, 90, 91). This tropism is thought to result from differences in the LTRs which facilitate the efficient transcription of the Mo-MuLV genome in T lymphocytes and the Fr-MuLV genome in erythroid precursors (92, 93, 94).

As indicated herein, the primary tropic determinant of the AIDS virus is the expression of the T4 protein on the surface of the target cell. *In vivo* infection is restricted to lymphoid cells and myeloid cells as well as brain cells: three populations which express T4. *In vitro* demonstrations indicate that the introduction of T4 into T4- human B lymphocytes and epithelial cells, cells which are not natural targets for AIDS virus, renders these cells susceptible to productive infection by AIDS virus.

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Example 1: Soluble T4 Fragments

Soluble T4 glycoprotein fragments are prepared using limited protease digestion from cell preparations. Alternatively, DNA expression vectors encoding T4 fragments which lack the transmembrane domain, a region containing neutral and hydrophobic residues, may be constructed and used to produce such T4 fragments. These fragments are soluble in aqueous solutions and contain leader (signal) sequences. When expressed in mammalian cells, these fragments are transported to the rough endoplasmic reticulum/golgi complex and eventually secreted from the cells.

Example 2: Treatment of AIDS Patients

Soluble T4 glycoprotein fragments as described in Example 1, typically in a pharmaceutically acceptable carrier, are administered to patients infected with a human immunodeficiency virus so as to bind to virus present in the subject's blood and other body fluids and block infection of T4+ cells in vivo. Alternatively or additionally, a patient's blood is cycled through a column containing either immobilized T4 glycoproteins or soluble T4 fragments so that the virus may be separated from the blood. Such measures permit the immune system to mount a more effective immunologic response against the virus, i.e., allow uninfected T4+ T cells to proliferate.

Soluble T4 fragments are used as a therapeutic, i.e., an inhibitor of extracellular and cell-cell spread of HIV infection. Applicants have shown that soluble T4 fragments inhibit in vitro HIV binding to, and infection of, T4+ target cells (see Example 4).

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Administration of soluble T4 fragments to persons infected with HIV inhibits extracellular spread of the virus infection. Additionally, fusion of HIV-infected T4+ cells and noninfected T4+ cells, which is also a route by which the virus spreads, are inhibited by administration of soluble T4 fragments.

Therefore, administration of soluble T4 fragments slows the course of disease, alleviates several symptoms associated with AIDS, and prevents occurrence of new pathologic changes.

Soluble T4 fragments, biochemically pure, aqueous soluble reagents, are used in combination with other reagents to assay for competitors of the T4-HIV interaction. Thus, soluble T4 fragments, in combination with HIV envelope proteins or biochemical mixtures containing HIV envelope proteins, are used to screen for inhibitors of viral binding.

20 Example 3: Production of Soluble T4 Fragments

A plasmid (pT4B) containing cDNA encoding the membrane-bound T4 protein has been isolated, characterized, and expressed in a variety of mammalian cell types (70). Soluble T4 fragments are produced in bacterial, yeast, insect, and mammalian systems. Because the native T4 protein likely folds in a complex manner and is glycosylated, expression in mammalian systems is preferred. Soluble T4 fragments are produced by truncating pT4B after the V<sub>4</sub>J<sub>4</sub> domain. Such DNA fragments terminate before the transmembrane segment, which begins at approximately nucleotide position 1264 (Figure 6).

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Purification and characterization of soluble T4 fragments is greatly enhanced by constructing a cell line which overexpresses the secreted protein fragment. Strategies which allow the overexpression of proteins have been employed in bacteria, yeast, insect, and mammalian systems. Inducible expression systems have also been employed in bacteria and yeast to overproduce proteins which may be toxic if constitutively expressed. Overexpression of soluble T4 fragments is accomplished by amplifying a soluble T4 expression vector, resulting in constitutive overexpression. The amplification of dihydrofolate reductase (dhfr) genes by growth in progressively increased concentrations of the drug methotrexate, an antagonist of dhfr, has been widely employed. Since the amplified unit is not limited to dhfr coding sequences, this approach results in the coamplification of sequences adjacent to them. Therefore, dhfr is used as a selectable marker and as a means of coamplifying newly introduced sequences. This strategy has been successfully employed to increase the expression of several different genes cotransformed with dhfr plasmids. An alternative amplification scheme involves cotransfection of the soluble T4 cDNA expression vector with the plasmid pDLAT-3 followed by a selection scheme as previously described (102).

Using recombinant DNA technologies, a vector expressing a secreted, soluble, extracellular fragment of T4 encoded by the human cDNA clone pT4B (70) is generated. Base pairs 1-1252 pT4B (see Figure 6) encode the leader peptide of T4 needed for the synthesis of a secreted protein, as well as the extracellular portion of T4 encompassing the four VJ-like domains (V1J1-V4J4), but not the transmembrane and cytoplasmic regions which

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anchor the protein in the membrane. This vector contains sequences encoding the extracellular portion of the T4 protein which contains the HIV binding domain. These sequences are placed downstream from the SV40 early region promoter. In addition, a TAA termination codon followed by the polyadenylation region of the bovine growth hormone gene is placed downstream from the truncated T4 cDNA to provide the signals necessary for termination of protein synthesis, transcription termination, and polyadenylation of the RNA transcript. The resulting soluble T4 minigene is then ligated to the mouse dihydrofolate reductase (dhfr) gene to generate a plasmid capable of being amplified after introduction into dhfr deficient (dhfr-) Chinese hamster ovary (CHO) cells.

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For example, the 1.3 kb EcoRI-BamHI fragment of pT4B, which contains the entire T4 coding sequence, is inserted between the SstI and BclI sites of the mammalian expression vector DSP (103) modified to contain the SV-40 early promoter and the bovine growth hormone polyadenylation sequence. Through the use of synthetic linkers, the HaeII (bp 124) - KpnI (bp 1252) fragment of pT4B is inserted between the KpnI and XbaI sites of the plasmid pUC18. A soluble T4 expression vector is created by ligating:

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1. a 0.95 kb BglII - SacI fragment of modified DSP which contains the 1.3 kb EcoRI-BamHI fragment of pT4B (this segment contains the SV40 early promoter,

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the T4 leader sequence, and the amino-terminal portion of the extracellular T4 sequence);

- 5        2. the 0.56 kb SacI - XbaI fragment of the pUC18 plasmid containing the HaeII-HpaII fragment of pT4B (this segment contains the carboxy terminal portion of the extracellular T4 sequence followed by a TAA termination codon inserted after valine 371 (Fig. 6)); and
- 10      3. the 2.48 kb BglII - XbaI fragment of modified DSP which contains the bovine growth hormone polyadenylation sequence.
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Finally, the 2.2 kb BglII - BamHI fragment from another modified DSP containing a mouse dhfr expression cassette ( $\beta$ -globin promoter - mouse dhfr coding region - SV40 polyadenylation region) flanked by BglII and BamHI sites, is inserted into the BamHI site of a plasmid to create a soluble T4 expression plasmid.

25      DXB-11, a clone of Chinese hamster ovary cells deficient in dhfr (104), is transfected with the soluble T4 expression plasmid. The DXB-11 transformants are then grown in F12 medium without hypoxanthine or thymidine containing 10% dialyzed fetal bovine serum. Clones are selected and subjected to stepwise increasing concentrations of methotrexate (mtx), an antagonist of dhfr, to select for stable transformants which have amplified the newly introduced dhfr gene and adjacent soluble T4 sequences.

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Culture supernatants of selected clones grown in mtk are subjected to radioimmunoprecipitation in order to detect soluble T4 fragments. Confluent cultures of selected clones are radiolaabelled with  $^{35}$ S-methionine and cysteine for 18 hrs and culture supernatants are immunoprecipitated with monoclonal antibodies specific for T4 (OKT4, OKT4A) as well as with control antibodies OKT8 and non-specific mouse IgG. The immunoprecipitates are subjected to SDS-polyacrylamide gel electrophoresis and exposed to film. A protein with an  $M_r$  of approximately 45 kd, the predicted size of a soluble T4 fragment, is specifically immunoprecipitated from culture supernatants by both OKT4 and OKT4A.

Conditioned medium (CM) is collected serum free from cultures of selected clones and clarified by low speed centrifugation and concentrated 10-fold. The concentrated sample is diluted 2-fold to reduce the salt concentration, adjusted to pH 5 and applied to S-Sepharose (Pharmacia). Soluble T4 fragments are retained on the resin at this pH and eluted with a salt gradient.

Similar approaches may be undertaken in bacteria, yeast and insects to produce soluble T4 fragments. In addition, fragments smaller in size than the one described herein, e.g. containing only the V<sub>1</sub>J<sub>1</sub> domain may be produced.

Example 4: Binding and Infectivity Assays Using Soluble T4 Fragments

Applicants tested the ability of a soluble T4 fragment prepared as described in Example 3 to compete with, and inhibit HIV binding to, T4+ cells. Serial

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dilutions of HIV virus were preincubated with serial dilutions of 10 X concentrated CM from selected soluble T4 fragment producing cells prior to addition to the target T4+ T cell line, CEM. HIV binding to CEM cells was quantitated by incubation with a FITC conjugated, 5 anti-HIV antibody followed by cytofluorometric analysis. CM from the selected soluble T4 fragment producing cell lines inhibited HIV binding to the surface of CEM cells in a dilution dependent manner, whereas no response was seen with CM from matched non-producer cells.

Applicants also tested the ability of soluble T4 fragments to inhibit HIV infectivity of T4+ cells in vitro. CM from a selected soluble T4 fragment producing cell line was added to cultures of PHA-stimulated T4+ T cells inoculated with serial dilutions of HIV. HIV replication was monitored in the cultures at days 4, 8 and 12 by the antigen capture assay described above. Soluble T4 fragments inhibited HIV infectivity at each time point by a factor of approximately 1 log.

Example 5: Preparation of Anti-Soluble T4 Fragment Antibodies

Eight week old Balb/c mice are injected intraperitoneally with 50 micrograms of a purified soluble T4 fragment of the present invention (prepared as described above) in complete Freund's adjuvant, 1:1 by volume. Mice are then boosted, at monthly intervals, with the soluble T4 fragment mixed with incomplete Freund's adjuvant, and bled through the tail vein. Immunoglobulin cuts of sera are generated by ammonium sulfate precipitation and specific anti-

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soluble T4 fragment antibodies are purified by affinity chromatography using an immobilized T4 fragment.

5 Example 6: Preparation of Soluble T4 Fragment Anti-Idiotypic Antibodies

Syngenic and congenic mice are injected intraperitoneally with 50 micrograms of a purified anti-soluble T4 fragment antibody, of the present invention (prepared as described above) in complete Freund's adjuvant and boosted with the anti-soluble T4 fragment antibody in incomplete Freund's adjuvant monthly. On days 4, 3, and 2 prior to fusion, mice are boosted intravenously with 50 micrograms of immunoglobulin in saline. Splenocytes are then fused with P3X63 AG8.653 non-secreting myeloma cells according to procedures which have been described and are known in the art to which this invention pertains. Two weeks later, hybridoma supernatants are screened for binding activity against anti-soluble T4 fragment antibodies by radioimmunoassay. Positive clones are then assayed for the ability to bind a human immunodeficiency virus envelope glycoprotein and AIDS virus. Alternatively, using the "one-step" procedure, mice are injected intraperitoneally with a soluble T4 fragment in complete Freund's adjuvant, boosted intravenously with the soluble T4 fragment in saline, and mice spleen cells fused with myelomas as above. Hybridoma supernatants are then assayed directly for soluble T4 fragment anti-idiotypic antibodies.

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What is claim d is:

1. A single-stranded nucleic acid molecule which encodes an amino acid sequence comprising at least a portion of a T4 glycoprotein.  
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2. A nucleic acid molecule of claim 1, wherein the amino acid sequence is capable of specifically forming a complex with a human immunodeficiency virus envelope glycoprotein.  
10
3. A nucleic acid molecule which is at least 90% homologous to the nucleic acid molecule of claim 2.  
15
4. A nucleic acid molecule of claim 3, wherein the amino acid sequence is soluble in an aqueous solution.  
15
5. A nucleic acid molecule of claim 1, wherein the T4 glycoprotein is a human T4 glycoprotein.  
20
6. A nucleic acid molecule complementary to the nucleic acid molecule of claim 1.  
25
7. A nucleic acid molecule of claim 6 labeled with a detectable marker.  
25
8. A DNA molecule of claim 1.  
30
9. A DNA molecule of claim 7 which comprises at least a portion of the genomic DNA molecule represented by the restriction enzyme map shown in Figure 10.  
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10. A cDNA molecule of claim 1 which comprises at least a portion of the nucleic acid sequence shown in Figure 6.
- 5 11. A cDNA molecule of claim 4, which comprises at least a portion of the nucleic acid sequence shown in Figure 6.
- 10 12. An RNA molecule of claim 1.
13. A method for detecting a single-stranded nucleic acid molecule encoding an amino acid sequence which is at least a portion of a T4 glycoprotein, which comprises contacting single-stranded nucleic acid molecules with a nucleic acid molecule of claim 7 under conditions permitting hybridization of complementary single-stranded nucleic acid molecules and separating hybridized nucleic acid molecules so formed from single-stranded nucleic acid molecules to thereby detect a single-stranded nucleic acid molecule which encodes an amino acid sequence which is at least a portion of a T4 glycoprotein.
- 20 14. A method according to claim 13, wherein the single-stranded nucleic acid molecule encoding an amino acid sequence which is at least a portion of a T4 glycoprotein is a DNA molecule derived from chromosomal DNA.
- 25 15. A method according to claim 14, wherein the chromosomal DNA is derived from a cell from the group consisting of lymphoid, myeloid, and brain cells.
- 30 16. A method according to claim 15, wherein the lymphoid cell is a T cell.

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17. A method according to claim 15, wherein the lymphoid cell is a B cell.
- 5 18. A method according to claim 15, wherein the myeloid cell is a granulocyte.
- 10 19. A method according to claim 15, wherein the myeloid cell is a macrophage.
20. An amino acid sequence which comprises at least a portion of a T4 glycoprotein and encoded by the nucleic acid molecule of claim 1.
- 15 21. An amino acid sequence capable of specifically forming a complex with a human immunodeficiency virus envelope glycoprotein and encoded by the nucleic acid molecule of claim 2.
- 20 22. An amino acid sequence which is at least 90% homologous to the amino acid sequence of claim 21.
- 25 23. An amino acid sequence capable of specifically forming a complex with a human immunodeficiency virus envelope glycoprotein and soluble in an aqueous solution, encoded by the nucleic acid molecule of claim 4.
- 30 24. A peptide which comprises at least one amino acid sequence of claim 20.
25. A polypeptide which comprises at least two peptides of claim 24.

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26. An amino acid sequence of claim 23 useful as a therapeutic agent for the treatment of acquired immune deficiency syndrome.
- 5 27. An amino acid sequence of claim 26 which comprises the amino acid sequence shown in Figure 6 from at least amino acid -23 to at most amino acid +374.
- 10 28. An amino acid sequence of claim 27 which comprises the amino acid sequence shown in Figure 6 from at least amino acid +287 to at most amino acid +374.
- 15 29. An amino acid sequence of claim 27 which comprises the amino acid sequence shown in Figure 6 from at least amino acid +182 to at most amino acid +286.
- 20 30. An amino acid sequence of claim 27 which comprises the amino acid sequence shown in Figure 6 from at least amino acid +112 to at most amino acid +181.
- 25 31. An amino acid sequence of claim 27 which comprises the amino acid sequence shown in Figure 6 from at least amino acid +1 to at most amino acid +111.
- 30 32. A pharmaceutical composition which comprises the amino acid sequence of claim 26 and a pharmaceutically acceptable carrier.
- 35 33. A method for treating a subject infected with a human immunodeficiency virus which comprises administering to the subject an effective amount of the pharmaceutical composition of claim 32.

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34. A purified polypeptide encoded by the cDNA molecule of claim 10.
- 5 35. A vector which comprises the cDNA molecule of claim 10.
36. A vector of claim 35 which comprises a plasmid.
- 10 37. A vector of claim 35 which comprises a virus.
38. A host vector system for the production an amino acid sequence which is at least a portion of a T4 glycoprotein which comprises the plasmid of claim 36 in a suitable host.
- 15 39. A host vector system of claim 38, wherein the suitable host is a bacterial cell.
- 20 40. A host vector system of claim 39, wherein the bacterial cell is an Escherichia coli cell.
- 25 41. A host vector system of claim 38, wherein the suitable host is a eucaryotic cell.
42. A host vector system of claim 41, wherein the eucaryotic cell is a mammalian cell.
- 30 43. A host vector system of claim 41, wherein the eucaryotic cell is a yeast cell.
44. A host vector system of claim 38, wherein the suitable host is an insect cell.

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45. A method for producing an amino acid sequence which is at least a portion of a T4 glycoprotein which comprises growing the host vector system of claim 38 under suitable conditions permitting production of at 5 least a portion of a T4 glycoprotein and recovering the resulting portion of a T4 glycoprotein.

46. A host vector system for the production an 10 amino acid sequence which is at least a portion of a T4 glycoprotein which comprises the virus of claim 37 in a suitable host.

47. A host vector system of claim 46, wherein the 15 suitable host is a bacterial cell.

48. A host vector system of claim 47, wherein the bacterial cell is an Escherichia coli cell.

49. A host vector system of claim 46, wherein the 20 suitable host is an eucaryotic cell.

50. A host vector system of claim 49, wherein the eucaryotic cell is a mammalian cell.

25 51. A host vector system of claim 49, wherein the eucaryotic cell is a yeast cell.

52. A host vector system of claim 46, wherein the 30 suitable host is an insect.

53. A method for producing an amino acid sequence which is at least a portion of a T4 glycoprotein which comprises growing the host vector system of claim 46 under suitable conditions permitting production of at 35 least a portion of a T4 glycoprotein and recovering the resulting portion of a T4 glycoprotein.

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54. A substance capable of specifically forming a complex with the amino acid sequence of claim 23.
- 5 55. An antibody of claim 54.
56. A monoclonal antibody of claim 55.
57. A human monoclonal antibody of claim 56.
- 10 58. A vaccine useful for immunizing a human subject against acquired immune deficiency syndrome which comprises the monoclonal antibody of claim 56 and a pharmaceutically acceptable carrier.
- 15 59. A method for immunizing a human subject against a human immunodeficiency virus which comprises administering to the subject an effective immunizing amount of the vaccine of claim 58.
- 20 60. A substance capable of specifically forming a complex with the monoclonal antibody of claim 56.
- 25 61. A substance of claim 60 capable of additionally forming a specific complex with a human immunodeficiency virus envelope glycoprotein.
62. A substance of claim 61 which comprises a T4 glycoprotein anti-idiotypic antibody.
- 30 63. A pharmaceutical composition which comprises the T4 glycoprotein anti-idiotypic antibody of claim 62 and a pharmaceutically acceptable carrier.